From: s22
To: Streamlined Submission; s22
Cc: s22
Subject: FW: Potential application - Ivermectin - Statistical principles for clinical trial design [SEC=OFFICIAL]
Date: Friday, 21 May 2021 12:02:14 PM
Attachments: image001.png
image002.png
image003.png

FYI

Kind regards s22

s22
Strategic Lead – COVID-19 vaccine regulation
Medicines Regulation Division | Health Products Regulation Group
Australian Government Department of Health
s22
Location: TGA, 136 Narrabundah Lane Symonston
c/- PO Box 100, ACT 2606, Australia

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From: s22
Sent: Friday, 21 May 2021 12:01 PM
To: s22 @cdd.com.au>
Cc: s22 @health.gov.au>; s22 @gmail.com>; s22 @health.gov.au>
Subject: RE: Potential application - Ivermectin - Statistical principles for clinical trial design [SEC=OFFICIAL]

Good morning s22

Just reflecting on our conversation the other day, and I thought the following international guideline may be helpful. This guideline has been adopted by the TGA and describes the statistical consideration for clinical trial design, and indeed the sample size for a clinical trial. I'm mindful that the clinical trials that you describe to me (in brief) may not be sufficiently powered (ie your sample size may be too small) to enable conclusions to be drawn about the efficacy of your proposed treatment. If you haven't already done so, you may wish to consider having your clinical trial design reviewed by a biostatistician, as this biostatistics report is one of several key considerations for the TGA when evaluating your application.

Statistical guideline for clinical trials: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002928.pdf

Further, grateful if you can please confirm who will be the sponsor for this application.

Kind regards s22

s22
Strategic Lead – COVID-19 vaccine regulation
Medicines Regulation Division | Health Products Regulation Group
Australian Government Department of Health
s22
Location: TGA, 136 Narrabundah Lane Symonston
c/- PO Box 100, ACT 2606, Australia

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From: s22 @cdd.com.au>
Sent: Wednesday, 19 May 2021 7:37 PM
To: s22 @health.gov.au>
Cc: s22 @health.gov.au>; s22 @gmail.com>
Subject: RE: Potential application - Ivermectin - Australian sponsor [SEC=OFFICIAL]

Hi s22

Thank you for speaking with me the other day.

I'm getting the data together and will send you information as per your points below about our Anti-Viral

Kind Regards,

s22
Director, Centre for Digestive Diseases
Director, Microbiome Therapeutics Institute

Centre for Digestive Diseases
s22
www.cdd.com.au
Level 1, 229 Great North Road, Five Dock NSW Australia 2046

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From: s22 @health.gov.au>
Sent: Monday, 17 May 2021 9:34 AM
To: s22 @benefel.com.au; s22 @cdd.com.au>
Cc: Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>; s22 @health.gov.au>
Subject: RE: Potential application - Ivermectin - Australian sponsor [SEC=OFFICIAL]

Good morning s22

While the TGA can organise a meeting with your team, my understanding is that no meeting has been scheduled at this time.

There are a couple of elements that often help with the scheduling of a preliminary meeting and would be required from your team. I highlighted some of these elements in my previous email, such as having an Australian sponsor for the submission. In TGA experience, scheduling a preliminary meeting with your team as well as your Australian sponsor (https://www.tga.gov.au/role-sponsor) is very beneficial for your team to gain a full understanding of the application process and nuances as it relates to COVID-19 medicines. Similarly, providing the TGA with some preliminary information of your medicine and its clinical trial progress also help us help you, for instance:

- brief overview of the medicine
- clinical trial progress (proposed and conducted to-date)
- overseas regulatory status (authorisations, under evaluation)
- proposed manufacturing supply (if known)
- list of questions for the TGA (note that the TGA may provide written responses in lieu of a meeting) or meeting agenda

Please let me know if you need any information on sourcing a regulatory agent, or indeed whether it is your intent to be the Australian sponsor.

-
Kind regards s22

s22
Strategic Lead – COVID-19 vaccine regulation
Medicines Regulation Division | Health Products Regulation Group
Australian Government Department of Health
s22
Location: TGA, 136 Narrabundah Lane Symonston
c/- PO Box 100, ACT 2606, Australia

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From: s22 @benefel.com.au s22 @benefel.com.au>
Sent: Friday, 14 May 2021 3:08 PM
To: s22 @health.gov.au>; s22 @cdd.com.au
Cc: Streamlined Submission <streamlinedsubmission@health.gov.au>
Subject: RE: Potential application - Ivermectin - The process begins - with Australia's safety in the face of Covid19 foremost on our minds - we race against the clock

REMINDER: Think before you click! This email originated from outside our organisation. Only click links or open attachments if you recognise the sender and know the content is safe.

Dear s22

Thank you for writing back to me.
While I remain in the loop, the key contact for this application will be s22 .
He has informed me that a Zoom meeting has already been organized.

I do see the email below outlines a template path forward which I trust your office will work through with s22 in a collective approach to bring essential life-saving options for Covid19 to the Australian people in the fastest and safest way possible.
I am sure the TGA and John Skerritt recognise this is no ordinary application for some pain relief medication and this medication and its indications being applied for may (will) play a key role in opening up Australia and significantly reducing the hospitalisations and death tolls of the pending wave of Covid19 infections that Australians face.

Aside from this discussion s22 , I can put you in direct touch with practicing world class Doctors in the US who are consisting reporting that 60% of the patients they are now treating for Covid19 symptoms are fully vaccinated; I say this so you will know that something more than Vaccines are needed in this fight against Covid19 and it is an urgent matter that Australia is prepared.

Every day saved in an efficient joint path through this process is critical in reducing the resultant hospitalization and death, as will be demonstrated in with the data that will be presented.
It need to be stated that the timing for this application is targeted to be within 2 weeks; delays beyond this will likely result in casualties that would have been avoided but for the length of the time of this application process.

s22 brings with him a team that should streamline this process, nevertheless, any assistance and guidance you can afford in your senior position in this process will play a critical role in the outcome for all Australians.

Again, thank you for writing and responding so quickly.

Best Regards

s22

From: s22 @health.gov.au]
Sent: Friday, 14 May 2021 1:14 PM
To: s22 @benefel.com.au; s22 @cdd.com.au
Cc: Streamlined Submission
Subject: Potential application - Ivermecton - further information [SEC=OFFICIAL]

Dear s22 ,

Thank you for contacting the TGA regarding your potential application for ivermectin. As the Strategic Lead for COVID-19 Vaccine Regulation, I head the TGA's COVID-19 medicines regulation coordination activities.

Applications for COVID-19 medicines are being treated with the greatest priority as part of the TGA's response to the COVID-19 global pandemic. The TGA is considering applications for COVID-19 medicines under the Prescription medicine provisional approval pathway (conditional approval) and/or the standard (full) registration pathway, based on the clinical trial data for individual applications.

Sponsor
Please note that an Australian sponsor is required for submission of an application to the TGA. The sponsor has certain legal obligations for the product under the *Therapeutic Goods Act 1989* and associated legislation. You may find it helpful to consider using a regulatory affairs consultant who is familiar with TGA processes, when you are preparing your submission dossier. The TGA is unable to provide recommendations for regulatory affairs consultants or sponsors, but in the first instance you may like to reach out to an regulatory affairs consultant via the appropriate industry associations for additional advice and assistance with Australian regulatory requirements. Sponsors are encouraged to seek independent legal advice to ensure that all legislative requirements are met.

Regulatory framework
The TGA website provides detailed guidance on the regulatory framework and on submitting an application to the TGA, including data requirements and fees. Please see the following:

- Australian Regulatory Guidelines for Prescription Medicines (ARGPM) includes:
  - general guidance
  - specific guidance on technical requirements
  - format of the Common Technical Document (CTD) dossier
  - data requirements across Modules 1-5 of the CTD dossier (i.e. Australian-specific information, quality, safety, efficacy)

- International scientific guidelines adopted in Australia. The TGA closely aligns its regulatory approaches to therapeutic products with those of comparable international regulatory counterparts wherever possible. In general terms, the TGA has similar expectations for submission dossiers and their evaluation, including following international scientific guidelines, however there are often national-specific requirements that a sponsor will also need to give consideration to.

- Provisional approval pathway - The sponsor must first apply for provisional approval determination based on the relevant eligibility criteria; if the criteria are met and the product is granted provisional determination, then an application can be made for provisional registration. Phase 1/2 Clinical trial data (Module 5) are usually insufficient to obtain a positive provisional determination outcome; in general, promising early Phase 3 data on safety and efficacy is required. Quality (Module 3) and nonclinical (Module 4) data should be of sufficient maturity before submitting an application for provisional registration. Sponsors should have defined clinical and manufacturing development plans for their COVID-19 medicine, and be able to provide details to the TGA. Sponsors should identify proposed supply chains and ensure GMP clearance applications for manufacturing sites are submitted to the TGA as early as possible.

- Fees and charges for prescription medicines - The relevant fees will depend on the proposed application type and proposed pathway for your product.

Pre-submission meetings

The TGA strongly recommends that sponsors hold a pre-submission meeting with the TGA prior to submitting an application for a COVID-19 medicine. The TGA can arrange meetings to discuss, in broad terms, the TGA's **regulatory framework and administrative processes** supporting COVID-19 medicine applications. Please contact streamlinedsubmission@health.gov.au with 'COVID-19 medicine' in the subject line, and state that you are seeking a general meeting with the TGA. A **formal technical pre-submission meeting** with TGA evaluators can be requested when there is a clear clinical development plan, and preferably once Phase 2/3 studies have been fully enrolled and initiated, and that there is a clear ability to transition from provisional registration to full registration

Please review the pre-submission meeting guidance and meeting request form. The TGA would be happy to arrange a pre-submission meeting once you have submitted your meeting request, including a technical briefing package that provides details on your proposed product:

- brief overview of the medicine
- clinical trial progress (proposed and conducted to-date)
- overseas regulatory status (authorisations, under evaluation)
- proposed manufacturing supply (if known)
- list of questions for the TGA (note that the TGA may provide written responses in lieu of a meeting)

I trust the above information addresses your queries.

Kind regards s22

s22
Strategic Lead – COVID-19 vaccine regulation
Medicines Regulation Division | Health Products Regulation Group
Australian Government Department of Health
s22
Location: TGA, 136 Narrabundah Lane Symonston
c/- PO Box 100, ACT 2606, Australia

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From: PEGG, Grant
To: s22 @bigpond.net.au; s22 @cdd.com.au
Cc: s22 @cdd.com.au; s22 @gmail.com; s22 @gmail.com; SKERRITT, John
Subject: Follow-up from 30 August meeting [SEC=OFFICIAL]
Date: Friday, 10 September 2021 1:39:00 PM
Attachments: Relevant guidance for consideration.docx
Comments on the clinical study synopsis.docx
Considerations when applying to register medicines.docx
image001.png

Dear s22 ,

Thank you for attending the videoconference on 30 August 2021 providing us with an update on the status of ivermectin triple therapy. At the meeting, we advised that we would provide you with some additional information and feedback.

Please find three documents attached which include:

- **Considerations when applying to register medicine/s for a new indication** – general points to consider across the various evaluation domains when preparing to make an application to the TGA
- **Relevant guidance for consideration** – links to regulatory guidance and information about Literature Based Submissions
- **Comments on the clinical study synopsis** – feedback on the draft study synopsis

We hope this information is useful.

Kind regards,

Grant

Dr Grant Pegg
Assistant Secretary

Medicines Regulation Division | Health Products Regulation Group
Prescription Medicines Authorisation Branch
Australian Government Department of Health
T: s22 | E: grant.pegg@health.gov.au
Location: Therapeutic Goods Administration GD 63
PO Box 100, Woden ACT 2606, Australia

Click here for the latest information on COVID-19

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Comments on the clinical study synopsis (draft 13.8.2021)

In the context that the proposed study may be used for a future regulatory application, the following preliminary comments are provided for the sponsor's consideration. Please note that these comments are not exhaustive or binding, nor should they be interpreted to pre-empt or replace the TGA's evaluation process that would be undertaken following receipt of an application. Sponsors are strongly encouraged to seek their own advice to ensure that regulatory requirements are met.

General comments

- The phase of the study should be clarified.
- For regulatory purposes, dose selection and proposed regimen would need to be justified.
- The power of the study should be carefully considered.
- The COVID-19 evidence taskforce definition of mild and moderate disease for inclusion in the study should be clarified. The taskforce recommends that patients with moderate disease (Sp02 >92% with up to 4L of O2) should be managed in hospital, when possible.
- Confirm that continuous telemonitoring is proposed (i.e. not intermittent).
- The study question should be clarified. Is it to evaluate the proportion of subjects who will achieve the endpoint following randomisation or the days required for subjects to achieve the endpoint following randomisation?

Comments on the primary endpoint

- The clinical justification/correlation of the primary endpoint (as a clinical test rather than outcome measure) should be carefully considered, in particular:
    - how the oxygen saturation level measured by telemetry/oximetry is closely corelated with clinical recovery
    - how subjects, for example with chronic lung diseases who may recover from COVID-19, but not be able to achieve oxygen saturation >98% will be managed
    - how this endpoint would translate to a potential indication for the medicines.
- The timepoint for the assessment of the primary endpoint should be made clear.
- Measurement precision should be carefully considered/controlled/validated given the choice of endpoint and the self-test proposal. How will user error be managed?
- In accordance with relevant guidance, the choice of clinically meaningful measures to assess effectiveness should be strongly considered.

Comments on the inclusion criteria

- The requirement of SpO2 < 95% could potentially exclude some mild symptomatic COVID-19 patients who may have normal SpO2 levels.
- Will the study stratify or focus on COVID-19 patients who are at high risk of developing severe COVID-19?
- Will the study include COVID-19 patients who are at an early stage of symptom onset (e.g. within 5-7 days of symptom onset) or COVID-19 patients with any duration after symptom onset?
- Will the study include people with any vaccination status (vaccinated and unvaccinated)?
- Will the study exclude patients who are concomitantly receiving another COVID-19 treatment/symptom management?

Considerations when applying to register medicine/s for a new indication

Below is a non-exhaustive list of items that should be considered by the sponsor prior to making a registration application.

General

Product sponsorship

The Australian sponsor is the legal entity responsible for the medicine/s in Australia. The sponsor is responsible for making the application for registration of the goods and also has legislated post-market responsibilities. Further details are available at: https://www.tga.gov.au/role-sponsor

Pharmacovigilance

Sponsors of registered goods also have pharmacovigilance responsibilities, these are outlined at: https://www.tga.gov.au/publication/pharmacovigilance-responsibilities-medicine-sponsors

Advisory Committee on Medicines

For an extension of indications application, subject to Delegate considerations, it is likely that advice would be sought from the independent clinical expert Advisory Committee on Medicines (ACM). The Delegate may seek advice from the ACM on a number of matters, including but not limited to clinical issues. ACM advice is considered by the Delegate when making a decision to register a product.

Dossier format

The dossier submitted must conform with the Common Technical Document format: https://www.tga.gov.au/publication/common-technical-document-ctd

From 1 November 2021, the Therapeutic Goods Administration (TGA) will begin a staged transition to Electronic Common Technical Document-only (eCTD-only) for all prescription medicines:
https://www.tga.gov.au/electronic-submissions

Quality and Manufacturing

- If domestic manufacturing sites are being used, these will need TGA licences. If a new manufacturing site is being proposed, obtaining a new licence may take some time.
- If overseas manufacturing sites are being used, the sponsor will need to demonstrate appropriate GMP. This can be achieved in multiple ways, depending on existing overseas regulatory requirements applicable to those sites (mutual recognition agreements with comparable regulators etc) but in some instances a TGA inspection may be required.
- Manufacturing quality would need to be assured within the application dossier, including batch release/QA specifications (where relevant).
- Further information about manufacturing requirements is available at: https://www.tga.gov.au/manufacturing-therapeutic-goods

Clinical

- Is the product's dose form and strength the same as a currently approved product?
- Are there data linking the proposed product with the currently registered product? This may include bioequivalence data depending on the nature of the application.
- Is it a single ingredient, composite pack or fixed dose combination?
- How does each component contribute to the product's efficacy and/or safety?
- Are there data on interaction between the components?
- What is the proposed dosage regimen and duration of treatment and is this supported by clinical trials?
- Have there been dose ranging studies to investigate different doses to select the optimal dose?
- Are there any drug interaction studies with other treatments?
- Are there well-designed and powered studies to determine efficacy, eg RCTs using the same products in the same combination and dose?
- Are there any non-supportive studies?
- Are there any ongoing studies and when are they expected to be completed?
- Who is the target population for treatment and what stage of disease is proposed for treatment?

Toxicology (non-clinical)

- Are there any appropriate animal models of COVID-19 infection to test the proposed treatment's efficacy?
- Are there toxicology data supporting the dose proposed for humans?
- Are there any *in vitro* or *in vivo* animal studies that could explain the proposed therapeutic product's mechanism of action?
- Are there any pharmacokinetic/toxicokinetic data from animal studies supporting the safety and pharmacology/efficacy studies?
- Are there any *in vitro* or *in vivo* studies that have looked at the potential pharmacological, pharmacokinetic and toxicological interactions with the ingredients in the proposed therapeutic product and with other drugs that patients may take?
- Have you completed any studies in animals that have investigated the effects on fertility, reproduction and development?
- Have you completed any toxicity studies in juvenile animals?
- Were safety-related studies conducted under GLP conditions? Do you have information on the active ingredients' batch numbers and do you know their impurity profiles?

Risk Management Plan

- It is likely that a Risk Management Plan (RMP) would be required, as is it is for other COVID-19 treatments. RMP requirements are detailed at: www.tga.gov.au/publication/risk-management-plans-medicines-and-biologicals

Guidance for Literature Based Submissions

- The TGA website includes information on literature based submissions (LBS) that covers pre-submission guidance, dossier requirements and systematic literature searches: https://www.tga.gov.au/publication/literature-based-submissions

- The requirements for each module are discussed on the TGA website. Please note that literature-based data sets are not suitable for the quality (Module 3) component of an application as published reports rarely include sufficient validation information. A conventional Module 3 dossier should be submitted for all applications with a quality component.

- Prior to lodging the Pre-submission Planning Form (PPF) for a LBS, the TGA will need to have reviewed and provided written agreement for the literature search strategy, including the databases to be searched, and the criteria for determining which search results are to be included/excluded in the application dossier.

- In situations where the methodology or regulatory approach needs further consideration/discussion, a pre-submission technical meeting is strongly encouraged. Questions about pre-submission issues can be directed to: streamlinedsubmission@health.gov.au

Relevant clinical guidance for consideration

Based on preliminary information presented by the sponsor, the following guidance is likely to be relevant to the application wholly, or in part:

- **Points to consider on application with 1. meta-analyses; 2. one pivotal study** (https://www.ema.europa.eu/en/documents/scientific-guideline/points-consider-application-1meta-analyses-2one-pivotal-study_en.pdf)

  In particular, section III.2 states:

  In cases where the confirmatory evidence is provided by one pivotal study only, this study will have to be exceptionally compelling, and in the regulatory evaluation special attention will be paid to:

  - The internal validity. There should be no indications of a potential bias.
  - The external validity. The study population should be suitable for extrapolation to the population to be treated.
  - Clinical relevance. The estimated size of treatment benefit must be large enough to be clinically valuable.
  - The degree of statistical significance. Statistical evidence considerably stronger than $p < 0.05$ is usually required, accompanied by precise estimates of treatment effects, i.e. narrow confidence intervals. The required degree of significance will depend on factors such as the therapeutic indication, the primary endpoint, the amount of supportive data and whether the alternative analyses demonstrating consistency are pre-specified. When the aim is to demonstrate non-inferiority, one study is more likely to be accepted if the lower 95% confidence bound is well away from the non-inferiority margin.
  -Data quality.

- Internal consistency. Similar effects demonstrated in different pre-specified sub-populations. All-important endpoints showing similar findings.
- Centre effects. None of the study centres should dominate the overall result, neither in terms of number of subjects nor in terms of magnitude of effect.
- The plausibility of the hypothesis tested

- **Guideline on clinical development of fixed combination medicinal products (**https://www.tga.gov.au/sites/default/files/ewp024095enfin.pdf)

  In particular, section 6 states:

  As a general rule, the choice of each substance in the fixed combination as well as the whole concept on which the rational for the fixed combination is based have to be fully justified; this can be achieved by taking into account mode(s) of action, pharmacokinetics, and treatment recommendations for a given clinical setting.

- **COVID-19: Developing Drugs and Biological Products for Treatment or Prevention Guidance for Industry (**https://www.fda.gov/media/137926/download))

  In particular, part III.A.2 states:

  FDA strongly recommends that drugs to treat COVID-19 be evaluated in randomized, placebo-controlled, double-blind clinical trials using a superiority design. In some situations, use of an active control with a superiority or noninferiority design may be appropriate.

  and part III.A.3 states:

  The drug development program should evaluate the effect of the investigational drug relative to placebo or an active comparator on clinically meaningful aspects of the disease. The relevance and appropriateness of measures may depend on the mechanism of action of the drug, the population studied, the clinical setting, the phase of drug development, and/or baseline disease severity (see Appendix A).

- It is also recommended that the sponsor review the various other adopted guidelines for prescription medicine applications which may be relevant to the application at: https://www.tga.gov.au/ws-sg-index

From: s22
To:
Cc: s22 SIMPSON, Andrew
Subject: RE: Preliminary TGA Meeting - Topelia Australia Ltd - Ivermectin triple therapy for the treatment of COVID-19 caused by SARS-CoV-2 virus [CEPL-PROJECTS.FID116094] [SEC=OFFICIAL]
Date: Thursday, 7 April 2022 10:56:46 AM
Attachments: image003.png
image002.png
image005.png

Hi s22

Below is some guidance to address the questions posed to the TGA in your briefing document and some additional information relevant to the COVID-19 applications.

The TGA is considering applications for COVID-19 medicines under the Prescription medicine provisional approval pathway (conditional approval) and/or the standard (full) registration pathway, based on the clinical trial data for individual applications. The sponsor must first apply for provisional approval determination; if the product is granted provisional determination, then an application can be made for provisional registration. Phase 1/2 Clinical trial data (Module 5) are usually insufficient to obtain a positive provisional determination outcome; in general, promising early Phase 3 data on safety and efficacy is required. Quality (Module 3) and nonclinical (Module 4) data should be of sufficient maturity before submitting an application for provisional registration. Sponsors should have defined clinical and manufacturing development plans for their COVID-19 medicine, and be able to provide details to the TGA. Sponsors should identify proposed supply chains and ensure GMP clearance applications for manufacturing sites are submitted to the TGA at the application stage.

Quality considerations

- Regarding whether TGA would accept less than the 6 months stability data at submission. Generally, the TGA prefers to evaluate longer term stability data, but depending on the availability and strength of supporting data, shorter-term data may be considered satisfactory for evaluation purposes in the interim.

Non-clinical considerations

- Guidelines on nonclinical studies consulted by the sponsor are applicable to the proposed combination. The Pre-submission guidance for literature based submissions (LBS) is relevant to both clinical and nonclinical data.
- Pharmacology studies (e.g. *in vitro* antiviral assays and in animal models of SARS-CoV-2 infection, RNA polymerase inhibition assay as claimed for zinc) with each ingredient and/or the combination would provide support to the proposed clinical indication. Pharmacology data for non-COVID-19 indications are not relevant to the proposed clinical use.
- It is recommended that the following be assessed (which can be based on literature if available):
    - the general toxicity and reproductive and developmental toxicity of the

combination
    - drug interactions between the 3 ingredients and between the drug combination and other drugs, which may be administered with Ziverdox
    - safety of ivermectin (all types of toxicity studies including repeat dose toxicity, genotoxicity, reproductive and developmental toxicity) given the higher dose and longer dosing duration than the approved clinical use

Clinical considerations

- Before applying for provisional determination, promising (interim) phase 3 results from the clinical trial to show the effectiveness of this ivermectin triple combination will be required. The following guideline describing appropriate clinical trial size may be helpful, as early conversations had indicated that some supporting clinical trials may not be sufficiently powered (ie your sample size may be too small) to enable conclusions to be drawn about the efficacy of your proposed treatment. Further, your team may wish to consider having your clinical trial design reviewed by a biostatistician, as this biostatistics report is one of several key considerations for the TGA when evaluating your application.
- Statistical guideline for clinical trials: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002928.pdf

Kind regards s22

s22
Strategic Lead – COVID-19 vaccine and treatment regulation
Medicines Regulation Division | Health Products Regulation Group
Australian Government Department of Health
s22
Location: TGA, 136 Narrabundah Lane Symonston
c/- PO Box 100, ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Thursday, 31 March 2022 9:59 AM
To: s22 @health.gov.au>
Cc: s22 @health.gov.au>
Subject: RE: Preliminary TGA Meeting - Topelia Australia Ltd - Ivermectin triple therapy for the

treatment of COVID-19 caused by SARS-CoV-2 virus [CEPL-PROJECTS.FID116094] [SEC=OFFICIAL]

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Hi s22

Thank you again for your time on the telephone yesterday and updated meeting request.

I'm just waiting to hear back from Topelia that the proposed time suits, and will then accept and forward the place holder to the relevant parties.

Have a lovely day.

Kind Regards
s22

s22
Senior Consultant, Regulatory, Quality & Compliance

From: s22 @health.gov.au>
Sent: Wednesday, 30 March 2022 10:54 AM
To: s22 @commercialeyes.com.au>
Cc: s22 @health.gov.au>
Subject: FW: Preliminary TGA Meeting - Topelia Australia Ltd - Ivermectin triple therapy for the treatment of COVID-19 caused by SARS-CoV-2 virus [CEPL-PROJECTS.FID116094] [SEC=OFFICIAL]

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Hi s22

I'm trying to confirm placeholder mtg with TGA, unfortunately there are some challenges with TGA attendees. I've tried to catch you be phone to discuss.

Is there an alternative date (other than the 8th April) that would also be possible for you?

Kind regards s22

s22
Strategic Lead – COVID-19 vaccine and treatment regulation
Medicines Regulation Division | Health Products Regulation Group
Australian Government Department of Health
s22

Location: TGA, 136 Narrabundah Lane Symonston
c/- PO Box 100, ACT 2606, Australia

The Department of Health acknowledges the traditional owners of country throughout Australia, and their continuing connection to land, sea and community. We pay our respects to them and their cultures, and to elders both past and present.

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From: s22 @commercialeyes.com.au>
Sent: Friday, 25 March 2022 4:53 PM
To: Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: Preliminary TGA Meeting - Topelia Australia Ltd - Ivermectin triple therapy for the treatment of COVID-19 caused by SARS-CoV-2 virus [CEPL-PROJECTS.FID116094]

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Dear Sir/Madam

On behalf of our client Topelia Australia Ltd, we would like to request a preliminary meeting with the TGA to discuss their development plans for the proposed ivermectin triple therapy for the treatment of COVID-19 caused by SARS-CoV-2 virus. The proposed product to be marketed under the trade name "Ziverdox", is a s47 .

The meeting is proposed for Friday 8th April 2022 at a time that suits the TGA representatives.

A background document is attached to provide the TGA with a general overview of the product; the proposed clinical, nonclinical and quality development program and data package; and a number of questions to facilitate the discussions. The outcome of the preliminary meeting will assist in finalising the proposed data set and regulatory strategy.

A formal meeting request including detailed pre-submission briefing package will be provided if a formal TGA pre-submission meeting is required in the future.

Please don't hesitate to contact me if you require any additional information to facilitate this meeting request.

Thank you in advance for your feedback.

Kind Regards

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A Commercial Eyes TGA Preliminary Meeting Background Document For

Topelia Ltd

Ivermectin, doxycycline and zinc citrate fixed s47

25 March 2022

Table of Contents

1. Purpose of the meeting 3
2. General Overview of the Product 3
Ivermectin 3
Doxycycline 4
Zinc Citrate 4
3. Development Program and Proposed Data Package 5
Quality 5
Nonclinical 6
Clinical 9
4. Proposed Regulatory Pathway 18
Application Details 18
5. Questions to the TGA 19
6. Consulted Guidelines 20
Nonclinical 20
Clinical 20
7. Appendices 21
8. References 22

1. Purpose of the meeting

Commercial Eyes Pty Ltd (CEPL) is submitting this background document on behalf of Topelia Ltd (Topelia) to facilitate discussions with the TGA in relation to their proposed medicine "Ziverdox", s47 s47 containing ivermectin, doxycycline and zinc citrate. The application is proposed to be submitted under the provisional registration pathway via a mixed (in-house studies and literature) application for the indication:

> *Ziverdox is indicated for the treatment of mild to moderate symptomatic patients diagnosed with COVID-19 caused by SARS-CoV-2 virus.*

Initial advice on the proposed medicine and regulatory strategy was sought from the TGA in August 2021. Subsequent concerns with the prescribing of ivermectin for the prevention or treatment of COVID-19 at the expense of vaccination, resulted in the TGA placing restrictions on the prescribing of oral ivermectin "New restrictions on prescribing ivermectin for COVID-19", 10 September 2021. The National COVID Clinical Evidence Taskforce (NCCET), the World Health Organisation (WHO) and the US Food and Drug Administration (FDA) also currently discourage the use of ivermectin monotherapy for COVID-19 treatment. The development program for Ziverdox has evolved taking the initial TGA advice and global concerns regarding the use of ivermectin monotherapy into consideration. This preliminary meeting is requested to seek further TGA guidance and feedback on the proposed regulatory strategy and data set, with specific questions posed as per section "5. Questions to the TGA" below.

This document is intended to provide the TGA with a general overview of the product; the clinical, nonclinical and quality development program and data package; and the proposed registration strategy. A detailed pre-submission briefing package will be provided if a formal TGA pre-submission meeting is required in the future.

2. General Overview of the Product

Topelia proposes to register Ziverdox as a s47 , comprising ivermectin s47 , doxycycline s47 and zinc s47 tablets for the treatment of COVID-19 caused by SARS-CoV-2 virus. The proposed dosage regimen is s47 .

The active ingredients in the tablet formulation are currently registered (ivermectin, doxycycline and zinc) and listed (zinc) on the ARTG and there are no novel excipients. Information relevant to the active ingredients in the proposed medicine is provided below, including specific considerations that relate to the development program.

Ivermectin

Ivermectin has been registered in Australia as an antiparasitic agent since 1999. It has been registered in an oral dose form since 27th August 1999 (Stromectol® 3 mg tablets; AUST R 181338; Merck Sharp & Dohme (Australia) Pty Ltd) for the treatment of anguillulosis and scabies. The dosage regimen is for 150 to 200 micrograms of ivermectin/kg body weight as a single dose (typically a

maximum dosing of 15 mg (Australian Product Information; Stromectol 2021)), depending on the condition.

The repurposing of ivermectin for the treatment of COVID-19 has been investigated in a large number of nonclinical and clinical studies given its apparent antiviral and anti-inflammatory properties. It is included in the formulation of Ziverdox given the body of evidence suggests it is safe and efficacious in the proposed indication.

The dose of ivermectin patients will receive is s47 . The nonclinical and clinical data sets will take into consideration the s47 , to inform the safety and efficacy of the proposed dosing regimen in the treatment of COVID-19.

Doxycycline

There are a number of products on the ARTG in tablet and capsule dose forms, containing 50 mg to 200 mg of doxycycline. The innovator product Doryx® capsules currently sponsored by Mayne Pharma International Pty Ltd was first included on the ARTG on 25th October 1991 and is registered for the treatment of infections caused by a number of micro-organisms. The maximum dose of Doryx (for primary and secondary syphilis) is 300 mg per day for 10 days.

Doxycycline is included in the formulation given the evidence suggesting efficacy against viral pathogens such as dengue fever, and regulation of pathways important in initial infection, replication and systemic response to SARS-CoV-2 (Yates et al. 2020).

s47 the safety profile of doxycycline is considered well established. The nonclinical and clinical development programs have been developed to specifically take into account the safety and efficacy of doxycycline in the proposed medicine for the treatment of COVID.

Zinc Citrate

Zinc in various salt forms is approved for use in many dosage forms in registered and listed medicines at different strengths. There doesn't appear to be any registered oral dose forms containing zinc s47

The rationale supporting the addition of zinc in the formulation of Ziverdox is its ability to inhibit the replication of RNA virus by the inhibition of RNA polymerase.

Zinc compounds are regulated as Schedule 4 for human internal use except:

a) in preparations with a recommended daily dose of 25 mg or less of zinc; or

b) in preparations with a recommended daily dose of more than 25 mg but not more than 50 mg of zinc when compliant with the requirements of the Required Advisory Statements for Medicine Labels (RASML).

s47 the

safety profile of zinc is considered well established. The nonclinical and clinical development programs have been developed to take into account the dose of zinc proposed in the regimen s47 to inform the safety and efficacy of zinc in the proposed medicine.

3. Development Program and Proposed Data Package

The proposed medicine "Ziverdox" is being developed as a s47 containing ivermectin, doxycycline and zinc citrate for the treatment of COVID-19. The rationale for using combination antiviral therapy is based on the growing understanding that intracellular infections – bacterial or viral, cannot be reliably cured using a single drug, in addition to prevent potential resistance if ivermectin is used alone (Hazan et al. 2021).

Initial advice on the proposed medicine and regulatory strategy was sought from the TGA in August 2021, and the development program has evolved taking the TGA advice into consideration.

When the TGA was initially approached regarding the medicine, Topelia proposed to register a s47

An overview of the proposed data package and development plan designed to obtain appropriate supporting data for the quality, efficacy and safety of the proposed medicine in the treatment of COVID-19 is described below.

Quality

The proposed medicine will be a s47 The active ingredients are currently registered in tablet dose form and the stability profiles established in these formulations. Compatibility of the three ingredients in the formulation will be determined during the development

There will be no new or novel excipients in the formulation.

As this is a conventional dose form, no specific challenges are anticipated in the development of the finished product with respect to chemistry, manufacturing and controls.

s47

s47

Nonclinical

The proposed nonclinical data set will be supported by literature and scientifically robust justifications as applicable. The table below provides a summary of whether a justification or literature reference is proposed to support the relevant nonclinical data set, with the rationale for the proposed action discussed under the specific headings below.

Table 1 Summary of the proposed nonclinical data set

Data requirements	Ivermectin	Doxycycline	Zinc	Combination
Pharmacology	s47			
Primary and secondary pharmacodynamics				
Safety pharmacology				
Pharmacodynamic drug interactions				
Pharmacokinetics				
Absorption, distribution, metabolism, excretion				
Pharmacokinetic drug interaction				
Toxicology				
Single and repeat dose toxicity				
Genotoxicity				
Carcinogenicity				
Reproductive and developmental toxicity studies				

J= justification
L= systematic literature review using search strategy approved by the TGA.

Pharmacology

Primary and Secondary Pharmacodynamics

s47

Safety Pharmacology

The mechanism of action (MOI) for the individual active ingredients does not include an overlap of the target organs; CNS for ivermectin, bacterial intracellular protein synthesis inhibition for doxycycline and gene expression for zinc, therefore a synergistic effect by the fixed dose combination that results in a safety concern in the target organs is considered to be unlikely. Given the unlikely interaction between the three ingredients and and the supporting clinical data proposed

for submission, s47

Pharmacodynamic Drug Interactions

Based on the available clinical data with the doublet regimen of ivermectin and doxycycline, the lack of overlap on the MOA for all three proposed ingredients, and the supporting clinical data proposed for submission, s47

Pharmacology Summary

s47

Pharmacokinetics

Absorption, Distribution, Metabolism, Excretion

The pharmacokinetic profiles of ivermectin and doxycycline are well known and described in the Product Information (PI) of currently registered products.

Zinc is absorbed throughout the whole small intestine (Maares and Haase 2020). Highest rates of absorption are unknown, believed to be in both the duodenum and jejunum. Distribution has been demonstrated to most body systems. Zinc is most readily metabolised in the small intestine lumen, then plasma, then less exchangeable zinc from other tissues, such as the liver. Zinc is excreted through a variety of pathways, including loss through faecal matter, urine, sweat, menstrual, semen, hair, nails, and skin.

Given the pharmacokinetics of each of the active ingredients are well established, and as combination studies are generally not recommended to support clinical trials or marketing in the individual agents have been tested according to current standards, s47

Pharmacokinetic Drug interactions

The approved ivermectin PI does not describe any clinical data on drug-drug interactions for ivermectin. There have been very rare post-marketing reports of increased normalisation ratio (time taken for blood to clot) when co-administered with warfarin. The PI for doxycycline also states reports of prolonged prothrombin time in patients taking warfarin and doxycycline.

Zinc deficiency is associated with bleeding and clotting abnormalities, and evidence exists that zinc serves as an effector in coagulation, anticoagulation and fibrinolysis depending on the microenvironment (Vu et al. 2013).

The pharmacokinetic drug-drug interaction between the three ingredients will be considered s47

Pharmacokinetic Summary

The pharmacokinetics section of the nonclinical data set will include scientific justifications together with literature targeted to:

- pharmacokinetic drug interactions of ivermectin, doxycycline and zinc in combination and alone
- assess the effect of concomitant use of anticoagulants

Toxicology

Single and repeat dose

The objective of single and repeat-dose studies is to inform the safety profile of a product prior to initial clinical studies. For combination products of well-established ingredients, single dose and repeated dose toxicity, as well as local tolerance investigations are not normally necessary.

s47

Genotoxicity

Ivermectin is negative for genotoxicity (Australian Product Information; Stromectol 2021).

There is no data reported for genotoxicity of doxycycline in the AU PI and the genotoxicity potential for zinc at the proposed dosage is unknown. It is proposed that a systematic literature search be undertaken to inform the genotoxic potential of doxycycline and zinc.

Carcinogenicity

According to the Stromectol PI there have been no carcinogenicity studies in humans with ivermectin. Ninety-four and 105 week carcinogenicity studies on mice and rats respectively were conducted with the closely related compound abamectin and were negative at up to 8 mg/kg/day in mice and up to 2 mg/kg/day in rats.

There are no carcinogenicity studies with doxycycline reported in the PI however, doxycycline has a long history of use at the dosages and duration of treatment proposed for Ziverdox. Zinc has been reported to not represent a carcinogenic risk to humans (Leonard et al. 1986).

s47

Reproductive and developmental toxicity studies

There are no data available on the effects of fertility for either ivermectin, doxycycline or zinc in Australian PIs.

In Australia, Ivermectin has a pregnancy Category B3, and doxycycline has a pregnancy Category D with use in pregnancy (16 weeks post conception) and use in lactation contraindicated. There is no corresponding PI for zinc therefore the pregnancy category could not be ascertained. A review of the information presented in eMIMS reveals several zinc supplements are classified as pregnancy Category D, consistent with the FDA directive for oral zinc.

Based on the information above, the use of Ziverdox in pregnancy (16 weeks post conception) will be contraindicated and contraindicated in lactation. s47 and exposure to the medicine will be managed by the pregnancy classification in the Product Information (PI).

Toxicity Summary

The nonclinical dataset will include scientifically robust justifications together with literature to support the following safety aspects of Ziverdox:

- overall safety profile of the triple therapy
- adverse event profile at proposed doses of ivermectin and zinc s47
- genotoxic potential of zinc and doxycycline

Clinical

The clinical data set is proposed to be supported by:

- Real world data written into study report format where applicable
- Literature as identified in a systematic literature review
- The clinical trial study protocol for the proposed Phase 3 clinical study (CDD20/05)
- A commitment to provide an interim analysis of the Phase 3 study results.

Scientifically robust justifications for the omission of data will also be included as applicable.
The clinical dossier to be submitted is therefore anticipated to include sufficient data to enable the TGA to weigh up the benefits and risks of the proposed medicine.

Early Treatment in COVID-19 with ITT – Real world data studies

Borody and Clancy 2021 - Ivermectin Triple Therapy

(Borody and Clancy 2021) present and analysed the data from pre-hospital treatment of Covid-19 patients in Sydney and Melbourne, Australia, using an ivermectin-doxycycline-zinc combination with observations on symptom resolution and oximetry. The data is presented on 600 patients with positive PCR symptomatic Covid-19 that were treated with a combination of ivermectin (24 mg per day), doxycycline (100 mg bd), and zinc (50 mg per day), for 10 days generally within 72 hrs of obtaining a positive PCR test for Covid-19, s47 . This was carried out
during the height of the COVID-19 pandemic infection by a group of Australian doctors [mainly general practitioners] using a protocol distributed to them but without CRO oversight. They prospectively prescribed ivermectin triple therapy (ITT) for patients with mild-moderate COVID-19 based on the Bellberry-approved lower-dose protocol so obtaining 'real world' data.

They treated over 600 consecutive patients with ITT prescribed 'off label' between June and September of 2021. A retrospective medical record review is proposed to collate the outcomes of

these patients. A new ethics approval is being sought for the study s47). The draft protocol for the study is provided in Appendix 1. This GP study was published on TrialSite News and was not peer reviewed (Borody and Clancy, 2021).

It reports treatment of a total of 600 patients with positive PCR tests using a ten-day course of ITT, which was fully completed in over 90% of those treated. Adverse events from the therapy were reported by 7% of patients, mainly minor gut symptoms of nausea, diarrhoea and heartburn. No patient stopped therapy due to adverse drug events and no patient discontinued therapy as a result of drug side effects. Two visited the hospital for less than 24 hours following a transient arrhythmia and then went home, while five were admitted to hospital (0.83%). There were no deaths. In an equivalent control group of 600, not treated with ITT, 70 were admitted to hospital (11.5%), with 6 deaths (1%). Chi-squared analysis of hospitalisation rate shows a significant decrease ($P<0.001$) in the presence of ivermectin triple therapy intervention. The control data was from contemporary infected subjects in Australia obtained from published Covid Tracking Data.

More detailed preliminary data from a subgroup in hotel and home quarantine was available for a assessment of symptoms and oxygenation status. This cohort of 126 was assessed for 10 symptoms according to a visual analogue scale (ranging from "0" for no symptoms, to "10" for most severe symptoms).

The 126 patients had a median age of 38 (range: 17-94). At least one symptom score was available for 125 of patients at baseline. Four patients did not report symptoms pre-treatment. All patients were classified as mild to moderate on the WHO ordinal scale pre-treatment. Fatigue, cough and muscle pains were the most commonly reported symptoms. Whilst fatigue and headaches were most severe. Of the patients with available pulse oximetry readings pre-treatment, the median oxygen saturation was 95% (71-99). Patients began treatment with ITT therapy within an average of 3.4 days (0-12) of diagnosis of Covid-19. Ten patients (8%) did not complete the full 10 days of treatment. Four patients did not complete the treatment due to worsening of disease, one did not complete due to improvement in symptoms, one did not complete due to adverse effects and the remaining four were stopped for unknown reasons.
In this cohort, eight (6%) of patients progressed to severe illness, critical illness; hospitalisation or intubation. Of the eight patients that progressed to severe illness during the study period, five required hospitalisation, four required oxygen support and two required intubation. No patients in this cohort died.

The majority of patients experienced improvement in symptoms (98%) post treatment with 35% reporting complete resolution of symptoms (Table 2). Symptoms that were most persistent were loss of taste and smell, cough, and fatigue. Overall, there was a significant reduction in symptom score from a median total symptom severity score of 37 (range: 0-100) at the onset of treatment to 3 (range: 0-62) at the conclusion of treatment ($P<0.0001$). Of the patients with available pulse oximetry readings post-treatment, the median oxygen saturation improved from 95% (71-99) to 98% (95-100).

A total of seven adverse events were reported during the treatment period including heartburn (n=2), diarrhoea, vomiting, face rash, exacerbation of asthma and hypertension. As mentioned previously, eight patients progressed to more severe illness during the study period, but only five required hospitalisation, four required oxygen support and two required intubation.

Table 2 Pre and post treatment symptom severity and pulse oximetry data from quarantined individuals (N=126)

Symptom	Pre-Treatment	Post-Treatment	P-Value*			
	N	Mean (SD)	Median (Range)	N	Mean (SD)	Median (Range)	
Fever	114	4.2 (4.1)	3 (0-10)	114	0.1 (0.7)	0 (0-5)	<0.0001
Headache	116	5.8 (3.3)	6 (0-10)	116	0.5 (1.1)	0 (0-5)	<0.0001
Cough	120	5.2 (3.3)	5 (0-10)	120	1.1 (1.9)	0 (0-9)	<0.0001
Loss of Taste	111	4.1 (4.5)	2 (0-10)	111	1.8 (3.2)	0 (0-10)	<0.0001
Loss of Smell	112	4.4 (4.5)	2.5 (0-10)	112	2.0 (3.5)	0 (0-10)	<0.0001
Diarrhoea	115	2.3 (3.3)	0 (0-10)	115	0.2 (0.7)	0 (0-5)	<0.0001
Vomiting	114	1.0 (2.7)	0 (0-10)	114	0.03 (0.3)	0 (0-3)	<0.0001
Fatigue	118	6.6 (3.4)	8 (0-10)	118	1.4 (2.0)	0 (0-10)	<0.0001
Muscle Pains	118	5.4 (3.7)	5 (0-10)	118	0.6 (1.4)	0 (0-7)	<0.0001
Joint Pains	114	4.7 (3.9)	5 (0-10)	114	0.4 (1.2)	0 (0-7)	<0.0001
Total Symptom Severity	124	40.9 (26.1)	37 (0-100)	124	7.5 (10.3)	3 (0-62)	<0.0001
Oximetry Reading (%)	71	93.3 (5.0)	95 (71-99)	71	98.1 (1.1)	98 (95-100)	<0.0001

Symptoms were scored on a 0-10 scale with 0 being the absence of associated symptom and 10 being the most severe presentation as reported by the patient. Total Symptom Severity Score was a combined sum of all symptoms assessed and was scored out of 100. Oximetry Readings were scored out of 100%. N = the number of respondents and SD = Standard Deviation. *Non-parametric paired t-tests were performed.

The results indicated that the combination of ivermectin, doxycycline and zinc at the proposed doses appears to be safe and efficacious, and the results of the study are also consistent with Hazan *et al* described below, noting the lower doses in the Hazan study.

Zimbabwe Prospective Trial - Ivermectin Triple Therapy

Topelia holds clinical data from a series of investigations in Zimbabwe for 36 patients treated with the proposed fixed dose combination therapy. The results of this study in 34 severe Covid-19 patients have now been published though peer review is ongoing (Stone et al. 2022).

The dosing schedule for the investigations was initially 12 mg ivermectin on the day of admission and then on days 4 and 8, plus doxycycline (100 mg B.I.D.) and zinc sulfate (60 mg/day). This regimen was used up through December 2020, when the second pandemic wave emerged in Zimbabwe. At that time, additional evidence of safety and tolerability of this regimen supported further dose escalation to a standard ivermectin dose regimen of 12 mg daily for five consecutive days, with adjunct use of doxycycline and zinc sulfate continued at the doses noted. In some cases, for which this standard

treatment regimen did not yield significant clinical gains within a few days, even higher doses of ivermectin were used, in some cases as high as 100 mg for a single dose. Pre and post treatment SpO2 values have been tracked for 34 of these patients, and the results for changes in SpO2 from pre- and post- treatment are summarised in the tables below.

Table 3 Mean standard deviation of changes in SpO_2 from pre and post treatment for COVID-19 patients treated with ivermectin triple therapy

Pre-treatment SpO_2	at +12 hours	at +24 hours	at +48 hours
60 - 84	+12.8 ± 6.7	+11.4 ± 6.1	+11.7 ± 5.8
85 - 89	+5.2 ± 2.6	+6.1 ± 2.6	+6.2 ± 2.8
90 - 93	+2.8 ± 2.7	+3.7 ± 2.3	+3.9 ± 1.8

As shown in the table, those patients with lowest, mid-range and highest pre-treatment SpO_2 values all had mean SpO_2 increases at +12 hours indicating clinical benefit for the ivermectin combination treatment.

Transient adverse effects (AEs) such as blurred vision characteristic of high dose ivermectin often occurred at those dose levels, but no serious AEs associated with ivermectin were manifested in any patient.

Literature Data

There is an increasingly number of clinical studies being undertaken to investigate the safety and efficacy of ivermectin in the treatment of COVID-19. Completed, ongoing and planned studies cover a range of strengths of ivermectin and range of treatment combinations, including the proposed combination of ivermectin, doxycycline and zinc.

To supplement the real world data, a systematic literature search will be conducted to identify all studies that meet the relevant inclusion criteria to inform the safety and efficacy of the individual components and proposed fixed dose combination for the treatment of COVID-19 and the studies written up as per the TGA LBS guidelines.

It is noted that there are a large number of clinical studies that have commenced or are to be commenced according to the FDA clinical trial data base. The status of these studies will continue to be monitored and literature studies identified will be assessed as they become available.

The initial draft of the proposed literature search strategy is provided in Appendix 2 for TGA consideration. The search terms have been developed taking into consideration the development and current body of evidence as described above.

A preliminary literature search conducted up to January 2021 has identified a number of studies pertinent to the proposed application, and the body of evidence suggests that the use of ivermectin in the treatment of COVID-19 overall has a positive risk/benefit profile when used in combination with doxycycline and zinc.

A summary of the results of some studies identified in the preliminary search are provided below.

Bryant et al 2021 – Ivermectin Alone

A systematic review and meta-analysis by (Bryant et al. 2021) assessed the efficacy of ivermectin treatment in reducing mortality, in secondary outcomes, and in chemoprophylaxis, among people with, or at high risk of, COVID-19 infection. Their systematic review up to 25th April 2021, identified 24 random controlled trials involving a total of 3,406 participants meeting their review inclusion criteria. For trials of COVID-19 treatment, 16 evaluated ivermectin among participants with mild to moderate COVID-19 only; 6 trials included patients with severe COVID-19.

The meta-analysis by the authors of 15 included trials found that ivermectin reduced risk of death by an average of 62% (95% CI 27%–81%) compared with no ivermectin (average risk ratio 0.38, 95% confidence interval 0.19–0.73; n = 2438; I^2= 49%; moderate-certainty evidence); risk of death 2.3% versus 7.8% among hospitalised patients in this analysis, respectively.

Table 4 below summarises the findings of ivermectin treatment versus no ivermectin treatment.

Low-certainty evidence found that ivermectin prophylaxis reduced COVID-19 infection by an average 86% (95% confidence interval 79%–91%). In addition, low-certainty evidence suggested that there may be no benefit with ivermectin for “need for mechanical ventilation,” whereas effect estimates for “improvement” and “deterioration” clearly favoured ivermectin use.

Meta-analysis of 11 trials, assessing 1533 participants, found that there was no significant difference between ivermectin and control in the risk of severe adverse events (aRR 1.65, 95% CI 0.44–6.09; I2 5 0%; low certainty evidence, downgraded for imprecision and study design limitations).

Table 4 Summary of findings of ivermectin versus no ivermectin for treatment of COVID-19 in any setting below shows the overall improvement

Outcomes	Illustrative comparative risks* (95% CI) Assumed risk No ivermectin	Corresponding risk Ivermectin	Relative effect (95% CI)	No. of participants (studies)	Quality of the evidence (GRADE)
Death from any cause	78 per 1000 (all disease severity)	48 fewer deaths per 1000 (21–63)	RR = 0.38 (0.19–0.73)	2438 (15)	Moderate†
Recovery time to negative PCR test, in days	Absolute risks were not computed due to certainty of evidence being low and, in some cases, number of events being sparse		MD = −3.20 (−5.99 to −0.40)	375 (6)	Very low†‡§
Time to clinical recovery, in days (outpatients)			MD = −1.06 (−1.63 to −0.49)	176 (2)	Very low†‡§
Time to clinical recovery, in days (mild to moderate COVID-19 inpatients)			MD = −7.32 (−9.25 to −5.39)	96 (1)	Very low†¶
Time to clinical recovery, in days (severe COVID-19 inpatients)			MD = −3.98 (−10.06 to 2.10)	33 (1)	Very low†¶
Admission to ICU			RR=1.22 (0.75–2.00)	379 (2)	Very low¶,‖
Need for mechanical ventilation			RR=0.66 (0.14–3.00)	431 (3)	Low§,‖
Length of hospital stay, in days			MD= 0.13 (−2.04 to 2.30)	68 (1)	Very low†,¶
Admission to hospital			RR 0.16 (0.02–1.32)	194 (2)	Very low†,¶
Duration of mechanical ventilation	Not reported				
Improvement (mild to moderate COVID-19)*	635 improved per 1000	159 more per 1000 (from 51 more to 286 more)	RR 1.25 (1.08–1.45)	681 (5)	Low†,‡
Deterioration (any disease severity)	143 per 1000	93 fewer per 1000 (from 50 fewer to 116 fewer)	RR 0.35 (0.19–0.65)	1587 (7)	Low†,‡
Serious adverse events	7/867 (0.8%) had an SAE in ivermectin group and 2/666 (0.3%) in control		RR=1.65 (0.44–6.09)	1533 (11)	Low†,‡

*Only one study contributed to the "severe" COVID-19 subgroup and subgroup data were not pooled due to subgroup differences.
†Downgraded 21 for study design limitations.
‡Downgraded 21 for inconsistency.
§Downgraded 21 for imprecision.
¶Downgraded 22 for imprecision/sparse data.
‖Downgraded 21 for indirectness.

Hazan et al 2021 – Ivermectin Triple Therapy

The aim of the study conducted by (Hazan et al. 2021) was to investigate the effectiveness of ivermectin combination therapy in consecutive adult patients who had failed to qualify for the blinded controlled trial, who had refused hospitalisation and whose oxygen saturation (SpO_2) was low, sometimes as low as 73%. Twenty four of 26 entered subjects received the triple therapy regimen of ivermectin (12 mg on day 1, 4 and 8), doxycycline (100 mg B.I.D.) and zinc (25 mg per day B.I.D.). Two declined the treatment due to GP's advice. The treatment arm was compared to an

externally controlled trial (synthetic) arm which was calculated from the public Center for Disease Control (CDC) data base of COVID-19 subjects.

Endpoints were time from presentation to negative SARS-CoV-2 PCR; time from presentation to symptom resolution; progression to hospitalisation; and patient survival.

Hospitalisations and deaths were significantly fewer (p<0.002 or 0.05, respectively) than in background-matched controls from the CDC database (***Figure 1***). All 24/26 treated subjects resolved symptoms in 11 days on average, and oxygen saturation improved in 24hrs (87.4% to 93.1%, p=0.001) (***Figure 2***). The two patients who declined treatment died from Covid-19.

Figure 1 Survival and Hospitalisation Rate

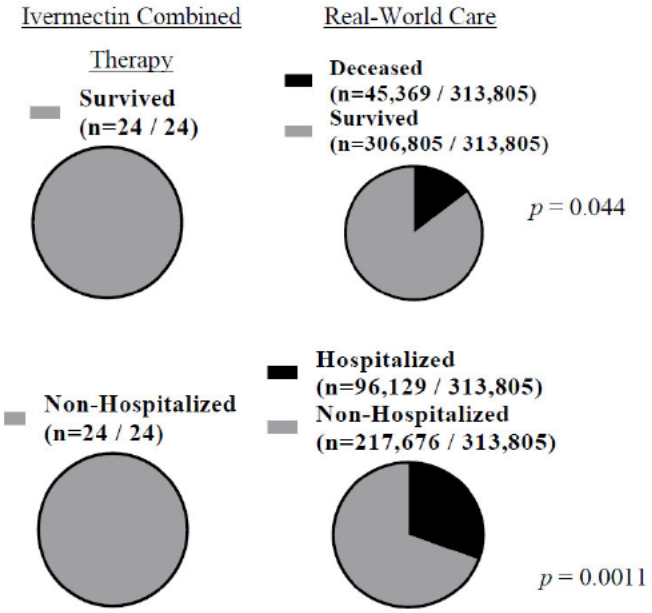
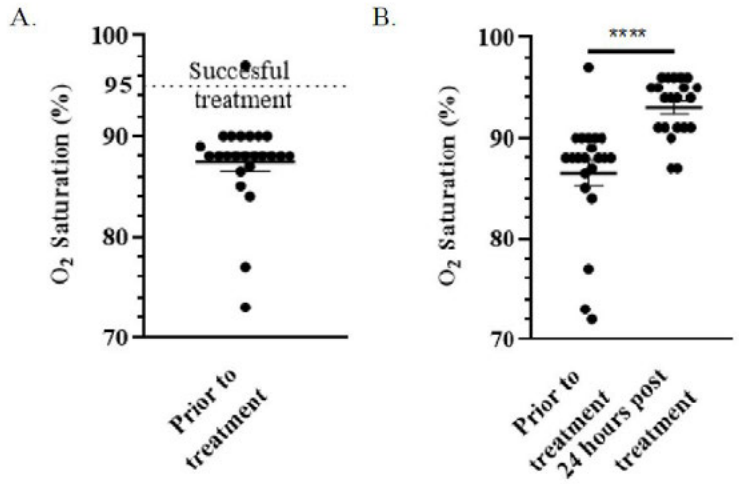


Figure 2 O_2 Saturation



A. O_2 saturation prior to treatment was significantly ($p < 0.05$) less than 95%, the defined successful treatment reached by all subjects.
B. O_2 saturation significantly increased in subjects 24 hours after treatment (paired *t*-test, $p < 0.001$, only subjects with data before and after treatment included). O_2 saturation continued to rise, and until the defined cure of greater than 95% O_2 saturation.

Adverse effects were rare. Dizziness appears to be the only adverse event reported by the authors and this occurred in one patient. The adverse event did not prevent the patient from continuing the trial treatment.

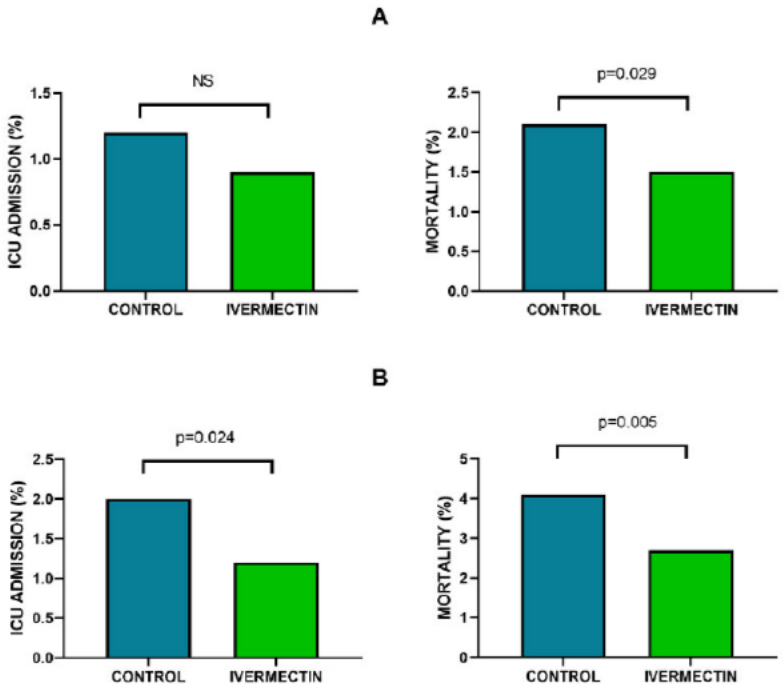
Mayer et al 2021 – Ivermectin Used Alone

In the study by (Mayer et al. 2022) the safety and efficacy of higher dose of ivermectin in COVID-19 patients was investigated in the MEURI Program, a monitored emergency use of unregistered and experimental interventions program in Argentina. Ivermectin was used alone and not as a Triple Therapy.

Adult patients with COVID-19 were administered ivermectin at a dose of 0.6 mg/kg/day for 5 days plus standard of care. Active pharmacosurveillance was performed for 21 days, and laboratory assessments including liver function tests were performed in a subset of patients. Frequency of Intensive Care Unit (ICU) admission and COVID-19-related mortality of subjects in the ivermectin intention to treat group were compared with that observed in inhabitants of the same province during the same period not participating in the program. From 21232 subjects with COVID-19, 3266 were offered and agreed to participate in the ivermectin program and 17966 did not and were considered as controls.

ICU admission was significantly lower in the ivermectin group compared to controls among participants ≥40 year-old (1.2% vs 2.0, odds ratio 0.608; p=0.024). Similarly, mortality was lower in the ivermectin group in the full group analysis (1.5% vs 2.1%, odds ratio 0.720; p=0.029), as well as in subjects ≥40 year-old (2.7% vs 4.1%, odds ratio 0,655; p=0.005).

Figure 3 ICU admission and mortality



A: Analysis of all subjects ≥18 year-old (C n= 17966; IVM-ITT n=3266)
B: Analysis of all subjects ≥40 year-old (C n= 9022; IVM-ITT n=1851)

A total of 2613 participants were included in the safety assessment. Of the patients included in the safety assessment, 567 participants reported 819 adverse events (AEs). 82 subjects (3.13 %) discontinued ivermectin due to adverse events and all AEs resolved after treatment discontinuation. The most common AEs were diarrhea, followed by visual disorders, 240 dizziness, abdominal pain, headache and nausea. It is noted that no comparison against the control arm was reported.

Phase 3 Clinical Study

Topelia plans to conduct a multi-centre, double blind randomised controlled trial of early treatment in patients to determine the effectiveness of the proposed fixed dose combination in curing COVID-19 infection; in improving COVID-19 symptoms; in reducing the % of patients progressing to severe or critical disease; and in preventing or minimising COVID-19 related morbidity and mortality. It is anticipated that the study will be completed by December 2022 contingent on availability of acute Covid-19 infected patients.

The Phase 3 clinical study (study number: CDD20/05) title is:

- A Multi-centre Double Blind Placebo Controlled Trial of Ivermectin Combination Therapy in patients diagnosed with Novel coronavirus (COVID-19) in the community setting

The study aims to recruit 480 adult patients, who have a positive test result for SARS-CoV-2 via nasopharyngeal swab derived RT-PCR and mild to moderate symptoms. Once consent has been obtained, the study participants who meet all the eligibility criteria, will be randomised to receive either the active treatment or placebo.
The objectives of the study are as follows.

Primary objectives:

To determine the:

- Effectiveness of Ziverdox in curing COVID-19 infection assessed as % of patients having consecutive negative RT-PCR swabs (in quarantined and self-quarantined subjects at Day 7 and 8 and at Day 14 and 15 and in the elderly population in aged care facilities by day 20 where negative results can be slower to attain)
- Effectiveness of Ziverdox in improving COVID-19 infection symptoms at Day 7 and Day 14.
- % of Ziverdox treated patients who progress to severe or critical disease symptomatically
- Effectiveness of Ziverdox preventing or minimising COVID-19 related morbidity and mortality defined as % of ITT population progressing to hospitalisation and/or requiring mechanical intubation.

Secondary objectives:

- To assess the safety and tolerability of Ziverdox in patients with COVID-19 infection

An interim analysis of the results is built into the study protocol and will be provided to the TGA when available during the evaluation.
The final data from the Phase 3 study will be the confirmatory data for full registration.

Summary

The real world data with details of the proposed Phase 3 clinical study and commitment to provide the results of the interim analysis, together with supplementary literature will comprise the clinical section of the dossier.

4. Proposed Regulatory Pathway

The application for registration of Ziverdox is proposed to be submitted s47

Application Details

The application will be submitted in s47

Quality Dossier

s47

Nonclinical Dossier

s47

Clinical Dossier

The clinical data set is proposed to be supported by:

s47

The data will be presented in modules 2.5, 2.7 and 5 as applicable.

Risk Management Plan

It is acknowledged that an Australian RMP will be required for inclusion in the registration application, and this will be prepared as per the current TGA guidance "Risk management plans for medicines and biologicals; Australian requirements and recommendations".

5. Questions to the TGA

s47

6. Consulted Guidelines

Nonclinical

- TGA's Guidance 23: Nonclinical studies, Version 1.1, September 2014
- ICH guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals EMEA/CHMP/ICH/286/1995
- EMA's Guideline on the nonclinical development of fixed combinations of medicinal products EMEA/CHMP/SWP/258498/2005
- EMA's Guideline on the nonclinical documentation for mixed marketing authorization applications (CPMP/SWP/799/95)
- Toxicokinetics: A guidance for assessing systemic exposure in toxicological studies code

Clinical

- COVID-19: Developing Drugs and Biological Products for Treatment or Prevention Guidance for Industry **(**https://www.fda.gov/media/137926/download))
- Clinical Investigation of Medicinal Products for Long Term Use https://www.tga.gov.au/sites/default/files/vol3cc6aen.pdf
- Guideline on clinical development of fixed combination medicinal products **(**https://www.tga.gov.au/sites/default/files/ewp024095enfin.pdf)
- COVID-19: Developing Drugs and Biological Products for Treatment or Prevention Guidance for Industry **(**https://www.fda.gov/media/137926/download))
- M4E(R2) - Common technical document for the registration of pharmaceuticals for human use – Efficacy
- CPMP/EWP/560/95/Rev. 1 Corr.* Guideline on the Investigation of Drug Interactions
- ICH Topic E1 Population Exposure: The Extent of Population Exposure to Assess Clinical Safety
- CPMP/ICH/378/95 ICH Topic E4 - Note for Guidance on Dose Response Information to Support Drug Registration
- EMA/CHMP/158268/2017 Guideline on clinical development of fixed combination medicinal products
- Mandatory-requirements-effective-application-prescription-medicines-180215
- https://www.tga.gov.au/safety-information/covid-19
- COVID-19 treatments
- https://www.tga.gov.au/covid-19/covid-19-treatments
- COVID-19 treatments: Information for sponsors (industry)
- https://www.tga.gov.au/covid-19-treatments-information-sponsors-industry
- Guidance for Literature Based Submissions - The TGA website includes information on literature based submissions (LBS) that covers pre-submission guidance, dossier requirements and systematic literature searches. Prior to lodging the Pre-submission Planning Form (PPF) for a LBS, the TGA will need to have reviewed and provided written agreement for the literature search strategy, including the databases to be searched, and the criteria for determining which search results are to be included/excluded in the application dossier https://www.tga.gov.au/publication/literature-based-submissions

- FDA Considerations for the Use of Real-World Data and Real-World Evidence to Support Regulatory Decision-Making for Drug and Biological Products, Draft Guidance for Industry, DECEMBER 2021 https://www.fda.gov/regulatory-information/search-fda-guidance-documents/considerations-use-real-world-data-and-real-world-evidence-support-regulatory-decision-making-drug
- Points to consider on application with 1. Meta-analyses; 2. One pivotal study CPMP/EWA/2330/99 https://www.ema.europa.eu/en/documents/scientific-guideline/points-consider-application-1meta-analyses-2one-pivotal-study_en.pdf

7. Appendices

Appendix 1: Draft protocol for Study no: CCD21/C05

Appendix 2: Draft literature search strategy

8. References

Only references relating to the proposed clinical data package are provided. Other refererences are available upon request.

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Pages 28-60 have been removed as they are exempt from release according to section 47 of the FOI Act.

COMBINATION THERAPY FOR COVID-19 BASED ON IVERMECTIN IN AN AUSTRALIAN POPULATION

TrialSiteNews – October 19, 2021

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Background

The immediacy of "real-world" data in the pre-hospital treatment of Covid-19 using re-purposed drugs has been the keystone to the development of effective therapy in a pandemic situation. The sense of a "common-message database" for the therapeutic use of ivermectin (IVM) collated by clinicians over a time frame of little more than 12 months, involving patients and research workers across geographic and social lines, is extraordinary. From over 20 countries there are 63 controlled studies accepted for meta-analysis, that have included 47,500 subjects with 625 authors.

While this compelling database on the effectiveness of IVM would not be possible without "real-life" studies, at an individual level, these trials give additional value such as information on dosage and combination therapies. They also give confidence to local doctors and regulators as they seek optimal management strategies.

Here, we report successful pre-hospital treatment of Covid-19 patients in Sydney and Melbourne, Australia, using an ivermectin-doxycycline-zinc combination with an important set of observations on symptom resolution and oximetry.

The Study

Six hundred Australian residents with positive PCR symptomatic Covid-19 were treated with a combination of ivermectin (24 mg per day), doxycycline (100 mg bd), and zinc (50mg per day), for 10 days within 48 hrs of obtaining a positive PCR test for Covid-19. The treatment period was from June to September 2021. Seven percent (7%) were given additional vitamins and nutritional supplements. Only 7% identified adverse events from the therapy, mainly minor gut symptoms of nausea, diarrhoea and heartburn. No patient stopped therapy due to adverse drug events.

A subgroup in hotel and home quarantine was available for a more detailed assessment of symptoms and oxygenation status. This cohort of 126 was assessed for 10 symptoms according to a visual analogue scale (ranging from "0" for no symptoms, to "10" for most severe symptoms). Descriptive statistics are summarised in Table 1. P-Values were calculated using Wilcoxon matched-pairs signed-rank t-tests. Chi Squared analysis was used to analyse rates of hospitalization in the 600-patient cohort.

The Outcome

A total of 600 patients with positive PCR tests were treated with the ten-day course of "Ivermectin Triple Therapy" (ITT), which was fully completed in over 90% of those treated. None discontinued therapy as a result of drug side effects. Two visited the hospital for less than 24 hours following a transient arrhythmia and then went home, while five were admitted to hospital (0.83%). There were no deaths. In an equivalent control group of 600, not treated with triple therapy, 70 were admitted to hospital (11.5%), with 6 deaths (1%). Chi-squared analysis of hospitalisation rate shows a significant decrease ($P<0.001$) in the presence of

ivermectin triple therapy intervention. The control data was from contemporary infected subjects in Australia obtained from published Covid Tracking Data.
The subgroup of 126 in quarantine had more detailed documentation. They had an average age of 42 (range: 17-94). The results of symptom analysis are in Table 1. For the 10 symptoms analysed, 98% had a total symptom score severity reduction by the end of the treatment period (P< 0.0001). Symptoms that were most persistent were loss of taste and smell, cough, and fatigue. Oximetry readings in 71 subjects were consistent with mild disease in most (Table 1), though the range extended to the low 70's indicating severe disease in some individuals. At the completion of the 10 -day treatment programme, all subjects had normal oximetry readings.

Comment

This successful study of 600 consecutive subjects treated within 2 days of testing positive on PCR for Covid-19 infection, emphasises the value of early treatment. The results are consistent with the study by Hazan et al, further supporting the value of ITT therapy (ivermectin, doxycycline and zinc).[1] The current study differs from that of Hazan et al who successfully used lower doses of IVM and a shorter treatment duration. The current study also confirms the value of oximetry in monitoring response to therapy, with all hypoxic patients having normal oximetry levels following the treatment protocol. Few become hypoxic in the first 48 hours of symptoms. The rapid increase in oximetry values following IVM, noted by Hazan[1], confirms significant hypoxia was likely avoided in this treated group. Combining oximetry with visual analogue scales to monitor symptoms, is suggested as a valuable tool for future studies.

Visual analogue scale monitoring of symptoms of Covid-19 infection showed a fall from a median total symptom severity score of 37 (range: 0-100) at the onset of treatment to 3 (range: 0-62) at the conclusion of treatment (P<0.0001). The main persistent symptoms were loss of smell/taste, cough and fatigue, recognised to persist for longer periods, however, all displayed significant severity score reductions during the treatment period (P<0.0001). The impact of early treatment with ITT on subsequent "long Covid" will be an important question to pursue. Symptom resolution with untreated Covid-19 is generally 2-4 weeks, emphasising the value of visual analogue scale assessment.

The major differences in admission to hospital and death following ITT therapy compared to contemporary controls is consistent with the large and increasing body of data measuring the impact of ivermectin-combination therapy.[1–3] The results from this study invites comparison with recently announced data by Merck from a study of their re-cycled antiviral polymerase inhibitor, Molnupiravir. The Merck study with 385 treated patients involved a similar group to that treated in the current Australian study judged by near-identical hospital admission and mortality rates in both control groups. In the Merck study, the hospital admission rate was halved (7.3% of treated patients) with no deaths, similar to data for the "first cousin" of Molnupiravir, Favipiravir, which is used extensively in Russia. The important point is that the admission rate in the Merck study is 8-fold greater than the 90% reduction in hospital admissions recorded in the current ITT study (P<0.0001). The public health impact of these findings with a cheap, safe and available therapy, in terms of reduction of load on a health system that will be further stressed as country borders are re-opened, cannot be ignored.
The current study included "real-life" data on consecutive patients thus avoiding selection bias. Therapy was given in two states of Australia, involving over 30 front-line doctors. This compelling data reinforces the value of information from a range of sources and trial methodologies in assessing treatment options, especially in a pandemic situation. The number of patients in this report was capped at 600, as the Australian regulatory body (the TGA)

intervened in the middle of Australia's third Covid wave, to prohibit further prescription of ivermectin for Covid-19 by General Practitioners.

Table 1: Pre and Post Treatment Symptom Severity and Pulse Oximetry Data from Quarantined Individuals (N=126)

	Pre-Treatment			Post-Treatment			
Fever	114	4.2 (4.1)	3 (0-10)	114	0.1 (0.7)	0 (0-5)	<0.0001
Headache	116	5.8 (3.3)	6 (0-10)	116	0.5 (1.1)	0 (0-5)	<0.0001
Cough	120	5.2 (3.3)	5 (0-10)	120	1.1 (1.9)	0 (0-9)	<0.0001
Loss of Taste	111	4.1 (4.5)	2 (0-10)	111	1.8 (3.2)	0 (0-10)	<0.0001
Loss of Smell	112	4.4 (4.5)	2.5 (0-10)	112	2.0 (3.5)	0 (0-10)	<0.0001
Diarrhoea	115	2.3 (3.3)	0 (0-10)	115	0.2 (0.7)	0 (0-5)	<0.0001
Vomiting	114	1.0 (2.7)	0 (0-10)	114	0.03 (0.3)	0 (0-3)	<0.0001
Fatigue	118	6.6 (3.4)	8 (0-10)	118	1.4 (2.0)	0 (0-10)	<0.0001
Muscle Pains	118	5.4 (3.7)	5 (0-10)	118	0.6 (1.4)	0 (0-7)	<0.0001
Joint Pains	114	4.7 (3.9)	5 (0-10)	114	0.4 (1.2)	0 (0-7)	<0.0001
Total Symptom Severity	124	40.9 (26.1)	37 (0-100)	124	7.5 (10.3)	3 (0-62)	<0.0001
Oximetry Reading (%)	71	93.3 (5.0)	95 (71-99)	71	98.1 (1.1)	98 (95-100)	<0.0001

Symptoms were scored on a 0-10 scale with 0 being the absence of associated symptom and 10 being the most severe presentation as reported by the patient. Total Symptom Severity Score was a combined sum of all symptoms assessed and was scored out of 100. Oximetry Readings were scored out of 100%. N = the number of respondents and SD = Standard Deviation. *Non-parametric paired t-tests were performed.

Conclusion

In 'Real Life' this 10-day Ivermectin Triple Therapy reported here, appears to be a very safe, effective and inexpensive early antiviral treatment for Covid-19. Further detail from the more than 30 doctors involved in treating this patient group, will be used for a formal peer-reviewed publication.

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FOI 26-3132 - Document 3

American Journal of Therapeutics 28, e434–e460 (2021)

OPEN

Ivermectin for Prevention and Treatment of COVID-19 Infection: A Systematic Review, Meta-analysis, and Trial Sequential Analysis to Inform Clinical Guidelines

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Background: Repurposed medicines may have a role against the SARS-CoV-2 virus. The antiparasitic ivermectin, with antiviral and anti-inflammatory properties, has now been tested in numerous clinical trials.

Areas of uncertainty: We assessed the efficacy of ivermectin treatment in reducing mortality, in secondary outcomes, and in chemoprophylaxis, among people with, or at high risk of, COVID-19 infection.

Data sources: We searched bibliographic databases up to April 25, 2021. Two review authors sifted for studies, extracted data, and assessed risk of bias. Meta-analyses were conducted and certainty of the evidence was assessed using the GRADE approach and additionally in trial sequential analyses for mortality. Twenty-four randomized controlled trials involving 3406 participants met review inclusion.

Therapeutic Advances: Meta-analysis of 15 trials found that ivermectin reduced risk of death compared with no ivermectin (average risk ratio 0.38, 95% confidence interval 0.19–0.73; n = 2438; I^2 = 49%; moderate-certainty evidence). This result was confirmed in a trial sequential analysis using the same DerSimonian–Laird method that underpinned the unadjusted analysis. This was also robust against a trial sequential analysis using the Biggerstaff–Tweedie method. Low-certainty evidence found that ivermectin prophylaxis reduced COVID-19 infection by an average 86% (95% confidence interval 79%–91%). Secondary outcomes provided less certain evidence. Low-certainty evidence suggested that there may be no benefit with ivermectin for "need for mechanical ventilation,"

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The preprint of this review received no funding. This updated version was funded by the crowdfunding initiative https://www.gofundme.com/f/help-us-get-lifesaving-drug-approved-for-covid19

The authors have no conflicts of interest to declare.

T. A. Lawrie and A. Bryant cowrote the review; they also sifted the search and classified studies for inclusion and entered and checked the data in RevMan and performed analyses. Data extraction was divided among T. A. Lawrie, A. Bryant, and T. Dowswell. T. Dowswell and A. Bryant graded the evidence. E. J. Fordham prepared the text on ivermectin mechanisms, use in pregnancy, and among the elderly. S. R. Hill prepared the brief economic commentary. Clinicians S. Mitchell and T. C. Tham contributed to the interpretation of the evidence in the discussion and conclusions. All authors reviewed and approved the final version of the manuscript.

This article discusses off-label use of the FDA-approved medication ivermectin against COVID-19.

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FOI 26-3132 - Document 3

whereas effect estimates for "improvement" and "deterioration" clearly favored ivermectin use. Severe adverse events were rare among treatment trials and evidence of no difference was assessed as low certainty. Evidence on other secondary outcomes was very low certainty.

Conclusions: Moderate-certainty evidence finds that large reductions in COVID-19 deaths are possible using ivermectin. Using ivermectin early in the clinical course may reduce numbers progressing to severe disease. The apparent safety and low cost suggest that ivermectin is likely to have a significant impact on the SARS-CoV-2 pandemic globally.

Keywords: ivermectin, prophylaxis, treatment, COVID-19, SARS-CoV-2

INTRODUCTION

To date, very few treatments have been demonstrated to reduce the burden of morbidity and mortality from COVID-19. Although corticosteroids have been proven to reduce mortality in severe disease,[1] there has been little convincing evidence on interventions that may prevent disease, reduce hospitalizations, and reduce the numbers of people progressing to critical disease and death.

Ivermectin is a well-known medicine that is approved as an antiparasitic by the World Health Organization and the US Food and Drug Administration. It is widely used in low- and middle-income countries (LMICs) to treat worm infections.[2,3] Also used for the treatment of scabies and lice, it is one of the World Health Organization's Essential Medicines.[4] With total doses of ivermectin distributed apparently equaling one-third of the present world population,[5] ivermectin at the usual doses (0.2–0.4 mg/kg) is considered extremely safe for use in humans.[6,7] In addition to its antiparasitic activity, it has been noted to have antiviral and anti-inflammatory properties, leading to an increasing list of therapeutic indications.[8]

Since the start of the SARS-CoV-2 pandemic, both observational and randomized studies have evaluated ivermectin as a treatment for, and as prophylaxis against, COVID-19 infection. A review by the Front Line COVID-19 Critical Care Alliance summarized findings from 27 studies on the effects of ivermectin for the prevention and treatment of COVID-19 infection, concluding that ivermectin "demonstrates a strong signal of therapeutic efficacy" against COVID-19.[9] Another recent review found that ivermectin reduced deaths by 75%.[10] Despite these findings, the National Institutes of Health in the United States recently stated that "there are insufficient data to recommend either for or against the use of ivermectin for the treatment of COVID-19,"[11] and the World Health Organization recommends against its use outside of clinical trials.[12]

Ivermectin has exhibited antiviral activity against a wide range of RNA and some DNA viruses, for example, Zika, dengue, yellow fever, and others.[13] Caly et al[14] demonstrated specific action against SARS-CoV-2 in vitro with a suggested host-directed mechanism of action being the blocking of the nuclear import of viral proteins[14,15] that suppress normal immune responses. However, the necessary cell culture EC_{50} may not be achievable in vivo.[16] Other conjectured mechanisms include inhibition of SARS-CoV-2 3CLPro activity[17,18] (a protease essential for viral replication), a variety of anti-inflammatory effects,[19] and competitive *binding* of ivermectin with the viral S protein as shown in multiple *in silico* studies.[20] The latter would inhibit viral binding to ACE-2 receptors suppressing infection. Hemagglutination via viral binding to sialic acid receptors on erythrocytes is a recently proposed pathologic mechanism[21] that would be similarly disrupted. Both host-directed and virus-directed mechanisms have thus been proposed, the clinical mechanism may be multimodal, possibly dependent on disease stage, and a comprehensive review of mechanisms of action is warranted.

Developing new medications can take years; therefore, identifying existing drugs that can be repurposed against COVID-19 that already have an established safety profile through decades of use could play a critical role in suppressing or even ending the SARS-CoV-2 pandemic. Using repurposed medications may be especially important because it could take months, possibly years, for much of the world's population to get vaccinated, particularly among LMIC populations.

Currently, ivermectin is commercially available and affordable in many countries globally.[6] A 2018 application for ivermectin use for scabies gives a direct cost of $2.90 for 100 12-mg tablets.[22] A recent estimate from Bangladesh[23] reports a cost of US$0.60—US$1.80 for a 5-day course of ivermectin. For these reasons, the exploration of ivermectin's potential effectiveness against SARS-CoV-2 may be of particular importance

FOI 26-3132 - Document 3

for settings with limited resources.[24] If demonstrated to be effective as a treatment for COVID-19, the cost-effectiveness of ivermectin should be considered against existing treatments and prophylaxes.

The aim of this review was to assess the efficacy of ivermectin treatment among people with COVID-19 infection and as a prophylaxis among people at higher risk of COVID-19 infection. In addition, we aimed to prepare a brief economic commentary (BEC) of ivermectin as treatment and as prophylaxis for COVID-19.[25]

METHODS

The conduct of this review was guided by a protocol that was initially written using Cochrane's rapid review template and subsequently expanded to a full protocol for a comprehensive review.[26]

Search strategy and selection criteria

Two reviewers independently searched the electronic databases of Medline, Embase, CENTRAL, Cochrane COVID-19 Study Register, and Chinese databases for randomized controlled trials (RCTs) up to April 25, 2021 (see **Appendix 1–3**, **Supplemental digital content 1**, http://links.lww.com/AJT/A95); current guidance[25] for the BEC was followed for a supplementary search of economic evaluations. There were no language restrictions, and translations were planned to be performed when necessary.

We searched the reference list of included studies, and of two other 2021 literature reviews on ivermectin,[9] as well as the recent WHO report, which included analyses of ivermectin.[12] We contacted experts in the field (Drs. Andrew Hill, Pierre Kory, and Paul Marik) for information on new and emerging trial data. In addition, all trials registered on clinical trial registries were checked, and trialists of 39 ongoing trials or unclassified studies were contacted to request information on trial status and data where available. Many preprint publications and unpublished articles were identified from the preprint servers MedRχiv and *Research Square*, and the International Clinical Trials Registry Platform. This is a rapidly expanding evidence base, so the number of trials are increasing quickly. Reasons for exclusion were recorded for all studies excluded after full-text review.

Data analysis

We extracted information or data on study design (including methods, location, sites, funding, study author declaration of interests, and inclusion/exclusion criteria), setting, participant characteristics (disease severity, age, gender, comorbidities, smoking, and occupational risk), and intervention and comparator characteristics (dose and frequency of ivermectin/comparator). The primary outcome for the intervention component of the review included death from any cause and presence of COVID-19 infection (as defined by investigators) for ivermectin prophylaxis. Secondary outcomes included time to polymerase chain reaction (PCR) negativity, clinical recovery, length of hospital stay, admission to hospital (for outpatient treatment), admission to ICU or requiring mechanical ventilation, duration of mechanical ventilation, and severe or serious adverse events, as well as post hoc assessments of improvement and deterioration. All of these data were extracted as measured and reported by investigators. Numerical data for outcomes of interest were extracted according to intention to treat.

If there was a conflict between data reported across multiple sources for a single study (eg, between a published article and a trial registry record), we contacted the authors for clarification. Assessments were conducted by 2 reviewers (T.L., T.D., A.B., or G.G.) using the Cochrane RCT risk-of-bias tool.[27] Discrepancies were resolved by discussion.

Continuous outcomes were measured as the mean difference and 95% confidence intervalss (CI), and dichotomous outcomes as risk ratio (RR) and 95% CI.

We did not impute missing data for any of the outcomes. Authors were contacted for missing outcome data and for clarification on study methods, where possible, and for trial status for ongoing trials.

We assessed heterogeneity between studies by visual inspection of forest plots, by estimation of the I^2 statistic ($I^2 \geq 60\%$ was considered substantial heterogeneity),[28] by a formal statistical test to indicate statistically significant heterogeneity,[29] and, where possible, by subgroup analyses (see below). If there was evidence of substantial heterogeneity, the possible reasons for this were investigated and reported. We assessed reporting biases using funnel plots if more than 10 studies contributed to a meta-analysis.

We meta-analyzed data using the random effects model (DerSimonian and Laird method)[30] using RevMan 5.4.1 software.[27,31] The results used the inverse variance method for weighting.[27] Some sensitivity analyses used other methods that are outlined below and some calculations were performed in *R*[32] through an interface[33] to the *netmeta* package.[34] Where possible, we performed subgroup analyses grouping trials by disease severity, inpatients versus outpatients, and single dose versus multiple doses. We performed sensitivity analyses by excluding studies at high risk of bias. We conducted further post hoc sensitivity analyses using alternative methods to test the robustness of results in the presence of zero events in both arms in a number of trials[35] and estimated odds ratios [and additionally RR for the Mantel–Haenszel

FOI 26-3132 - Document 3

(MH) method] using a fixed effects model. The models incorporate evidence from single-zero studies without having to resort to continuity corrections. However, double-zero studies are excluded from the analysis; so, the risk difference was also assessed using the MH method as this approach can adequately incorporate trials with double-zero events. This method can also use a random-effects component. A "treatment-arm" continuity correction was used, where the values 0.01, 0.1, and 0.25 were added where trials reported zero events in both arms. It has been shown that a nonfixed continuity correction is preferable to the usual 0.5.[35] Other methods are available but were not considered due to difficulty in interpretation, sensitivity of assumptions, or the fact they are rarely used in practice.[36–40]

Trial sequential analysis

When a meta-analysis is subjected to repeated statistical evaluation, there is an exaggerated risk that "naive" point estimates and confidence intervals will yield spurious inferences. In a meta-analysis, it is important to minimize the risk of making a false-positive or false-negative conclusion. There is a trade-off between the risk of observing a false-positive result (type I error) and the risk of observing a false-negative result (type II error). Conventional meta-analysis methods (eg, in RevMan) also do not take into account the amount of available evidence. Therefore, we examined the reliability and conclusiveness of the available evidence using trial sequential analyses (TSA).[41–43] The DerSimonian–Laird (DL) method was used because this is most often used in meta-analytic practice and was also used in the primary meta-analysis.[30]

The TSA was used to calculate the required information size (IS) to demonstrate or reject a relative reduction in the risk (RRR) of death in the ivermectin group, as found in the primary meta-analysis. We assumed the estimated event proportion in the control group from the meta-analysis because this is the best and most representative available estimate. Recommended type I and II error rates of 5% and 10% were used, respectively (power of 90%),[43] powering the result on the effect observed in the primary meta-analyses. We did not identify any large COVID-19 trials powered on all-cause mortality, so powering on some external meaningful difference was not possible. Any small RRR is meaningful in this context, given the scale of the pandemic, but the required IS would be unfeasibly high for this analysis if powered on a small difference. The only reliable data on ivermectin in its repurposed role for treatment against COVID-19 will be from the primary meta-analysis. Therefore, assuming it does not widely deviate from other published systematic reviews, a pragmatic decision was therefore made to power on the pooled meta-analysis effect estimate for all-cause mortality a priori. This is more reflective of a true meaningful difference. We used a model variance-based estimate to correct for heterogeneity. A continuity correction of 0.01 was used in trials that reported zero events in one or both arms. The required IS is the sample size required for a reliable and conclusive meta-analysis and is at least as large as that needed in a single powered RCT. The heterogeneity corrected required IS was used to construct sequential monitoring boundaries based on the O'Brien–Fleming type alpha-spending function for the cumulative z-scores (corresponding to the cumulative meta-analysis),[43] analogous to interim monitoring in an RCT, to determine when sufficient evidence had been accrued. These monitoring boundaries are relatively insensitive to the number of repeated significance tests. They can be used to further contextualize the original meta-analysis and enhance our certainty around its conclusions. We used a two-sided test, so also considered futility boundaries (to test for no statistically significant difference) and the possibility that ivermectin could harm. Sensitivity analyses were performed excluding the trial of Fonseca,[44] which was a cause of substantial heterogeneity (but retained in the core analysis because it was at low risk of bias). Its removal dramatically reduced I^2 and D^2 (diversity) estimates, thus reducing the model variance-based estimate to correct for heterogeneity. Two further sensitivity analyses were performed using 2 alternative random effect models, namely the Biggerstaff–Tweedie (BT) and Sidik–Jonkman (SJ) methods.[43]

All outcomes have been assessed independently by 2 review authors (T.D. and A.B.) using the GRADE approach,[45] which ranks the quality and certainty of the evidence. The results of the TSAs will also form part of the judgment for the primary all-cause mortality outcome. The results are presented in a summary of findings table. Any differences in judgments were resolved by discussion with the wider group. We used Cochrane Effective Practice and Organisation of Care guidance to interpret the evidence.[46]

RESULTS

Search results and risk-of-bias assessment

The combined and preliminary deduplicated total was n = 583. We also identified 11 records from other sources (reference lists, etc). See PRISMA flow diagram for inclusion and exclusion details of these references (Figure 1).

FOI 26-3132 - Document 3

The supplementary search for the BEC identified 17 studies, of which 4 were retrieved in full. No full trial- or model-based economic evaluations (cost–utility analyses, cost–effectiveness analyses, or cost–benefit analyses) were identified.

Twenty-one trials in treatment and 2 trials in prophylaxis of COVID-19 met review inclusion. One further study[47] reported separate treatment and prophylaxis components; we label this study "Elgazzar" under both questions. In effect, there were 22 trials in treatment and 3 in prophylaxis. All of these contributed data to at least one review outcome and meta-analysis. Fifteen trials contributed data for the primary outcome for ivermectin treatment (death); 3 studies reported the primary outcome for prophylaxis (COVID-19 infection). Characteristics of included studies are given in Table 1. Seventeen studies[47–63] were excluded as they were not RCTs and we identified 39 ongoing studies[64–102] and 2 studies[103,104] are awaiting classification.

A risk-of-bias summary graph is given in Figure 2. Eleven studies[23,24,44,47,105,106–111] used satisfactory random sequence generation and allocation concealment. Two trials described satisfactory sequence generation, but it was unclear whether allocation was concealed.[112,113]

Ten trials reported adequate blinding of the participants/personnel and/or the outcome assessors.[23,24,44,105,107,109,110,111,113,114] The others were either unclear or high risk for blinding. We considered blinding to be a less important criterion for evaluation of evidence related to the review's primary outcomes, namely death and laboratory-confirmed COVID-19 infection, which are objective outcomes.

We did not consider publication on preprint web sites to constitute a risk of bias because all studies were scrutinized and peer reviewed by us during the review process and, where additional information was needed, we contacted the authors for clarification.

Main findings

Twenty-four RCTs (including 3 quasi-RCTs) involving 3406 participants were included, with sample sizes ranging from 24 to 476 participants. Twenty-two trials in treatment and 3 trials in prophylaxis met review inclusion, including the trial of Elgazzar et al, which reported both components. For trials of COVID-19 treatment, 16 evaluated ivermectin among participants with mild to moderate COVID-19 only; 6 trials included patients with severe COVID-19. Most compared ivermectin with placebo or no ivermectin; 3 trials included an active comparator (Table 1). Three RCTs involving 738 participants

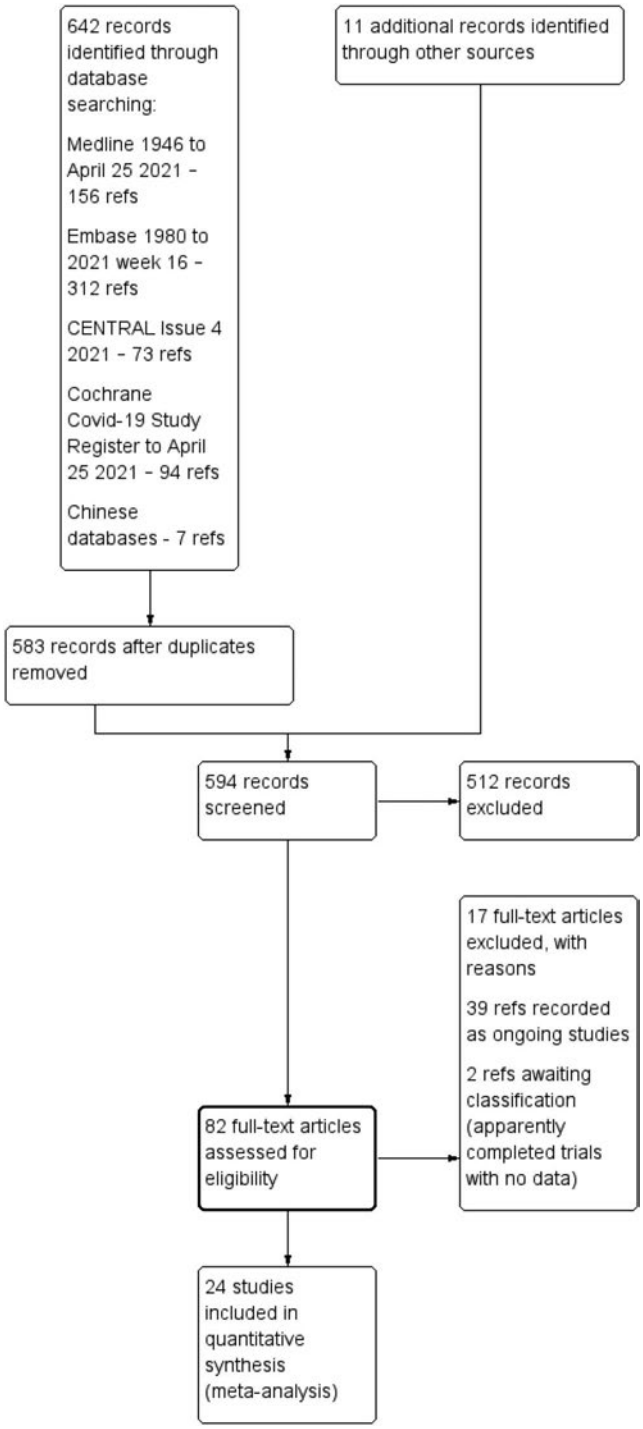


FIGURE 1. Study flow diagram from search on 25 April 2021.

were included in the prophylaxis trials. Most trials were registered, self-funded, and undertaken by clinicians working in the field. There were no obvious conflicts of interest noted, with the exception of two trials.[85,139]

FOI 26-3132 - Document 3

Table 1. Summary of study characteristics.

Study ID	Country	Design	Funding	Participants	Sample size	Ivermectin dose and frequency*	Comparator	Origin of data	Main outcomes reported
COVID-19 treatment studies									
Ahmed 2020[23]	Bangladesh	Double-blind	BPL(Pharma); Bangladesh, Canada, Sweden, and UK govt	Mild to moderate COVID (inpatients)	72	12 mg × 1 day or × 5 days (3 study arms)*	Placebo	Published in PR journal; emailed/ responded with data	Time to viral clearance (PCR –ve), remission of fever and cough within 7 days, duration of hospitalization, mortality, failing to maintain sats >93%, adverse events, PCR –ve at 7 and 14 days
Babalola 2020[105]	Nigeria	Double-blind	Self-funded	Asymptomatic, mild or moderate COVID (45 inpatients and 17 outpatients)	62	6 mg every 84 hrs × 2 wks (arm 1) or 12 mg every 84 hrs × 2 wks (arm 2)	Ritonavir/lopinavir	MedRxiv preprint: emailed/ responded with data. Paper accepted for publication	Time to PCR –ve, laboratory parameters (platelets, lymphocytes, clotting time), clinical symptom parameters
Bukhari 2021[135]	Pakistan	Open-label	None reported	Mild to moderate COVID (inpatients)	100	12 mg × 1 dose	SOC	MedRxiv preprint	Viral clearance, any adverse side effects, mechanical ventilation
Chaccour 2020[24]	Spain	Double-blind	Idapharma, ISGlobal, and the University of Navarra	Mild COVID (outpatients)	24	0.4 mg/kg × 1 dose	Placebo	Published in PR journal	PCR +ve at day 7, proportion symptomatic at day 4,7,14,21, progression, death, adverse events
Chachar 2020[112]	Pakistan	Open-label	Self-funded	Mild COVID (outpatients)	50	12 mg at 0, 12, and 24 hours (3 doses)	SOC	Published in PR journal	Symptomatic at day 7
Chowdhury 2020[136]	Bangladesh	Quasi-RCT	None reported	Outpatients with a +ve PCR (approx. 78% symptomatic)	116	0.2 mg/kg x1 dose*	HCQ 400 mg 1st day then 200 mg BID × 9 days + AZM 500 mg daily × 5 days	Research square preprint	Time to –ve PCR test; period to symptomatic recovery; adverse events
Elgazzar 2020[47]	Egypt	RCT	None reported	Mild to severe COVID (inpatients)	200	0.4 mg/kg daily × 4 days	HCQ 400 mg BID × 1 day then 200 mg BID × 9 days	Research square preprint: emailed/ responded with data	Improved, progressed, died. Also measured CRP, D-dimers, HB, lymphocyte, serum ferritin after one week of treatment
Fonseca 2021[44]	Brazil	Double-blind	Institution-funded	Moderate to severe (inpatients)	167	14 mg daily × 3 days (plus placebos × 2 additional days)	HCQ—400 mg BID on day 0 then daily × 4 days; CQ -450 mg BID day 0 then daily × 4 days	Prepublication data/ manuscript in progress obtained via email	Death, invasive mechanical ventilation
Gonzalez 2021[137]	Mexico	Double-blind	Institution-funded	Moderate to severe (inpatients)	108	12 mg × 1 dose	Placebo	MedRxiv preprint	Length of hospital stay, invasive mechanical ventilation, death, time to negative PCR
Hashim 2020[138]	Iran	Quasi-RCT	None reported	Mild to critical (inpatients)	140	0.2 mg/kg × 2 days* Some had a 3rd dose a week later	SOC	MedRxiv preprint	Death, mean time to recovery, disease progression (deterioration)
Krolewiecki 2020[106]	Argentina	Open-label	None reported	Mild to moderate (inpatients)	45	0.6 mg/kg/d × 5 days	Placebo	Research Gate and SSRN preprints	Viral load reduction in respiratory secretions day 5, IVM concentrations in plasma, severe adverse events
Lopez-Medina 2021[85]	Columbia	Double-blind	Institution-funded	Mild (outpatients)	476	0.3 mg/kg elixir × 5 days	Placebo	Published in a PR journal	Resolution of symptoms within 21 days, deterioration, clinical condition, hospitalization, adverse events
Mahmud 2020[107]	Bangladesh	Double-blind	None reported	Mild to moderate COVID (inpatients)	363	12 mg × 1 dose*	Placebo + SOC	Data published on clinical trial registry and clarification obtained via email	Improvement, deterioration, late clinical recovery, persistent PCR test +ve

(*Continued on next page*)

FOI 26-3132 - Document 3

Table 1. (*Continued*) Summary of study characteristics.

Study ID	Country	Design	Funding	Participants	Sample size	Ivermectin dose and frequency*	Comparator	Origin of data	Main outcomes reported
Mohan 2021[110]	India	Double-blind	Institution-funded	Mild to moderate	152	12 mg or 24 mg elixir × 1 dose	Placebo	MedRxiv preprint research	Conversion of RT-PCR to negative result, decline of viral load at day 5 from enrollment
Niaee 2020[108]	Iran	Double-blind	Institution-funded	Mild to severe COVID	180	0.2 mg/kg × 1 and 3 other dosing options) ~ 14 mg tablet†	Placebo	Research Square preprint	Deaths, length of stay, biochemical parameters
Okumus 2021[115]	Turkey	Quasi-RCT	None reported	Severe COVID	66	0.2 mg/kg × 5 days	SOC	Prepublication data/ manuscript in progress obtained via email	Clinical improvement, deterioration, death, SOFA scores
Petkov 2021[139]	Bulgaria	Double-blind	Pharma-funded	Mild to moderate COVID	100	0.4 mg/kg × 3 days	Placebo	Prepublication data obtained from another source	Rate of conversion to PCR negative
Podder 2020[140]	Bangladesh	Open-label	Self-funded	Mild to moderate (outpatients)	62	0.2 mg/kg × 1 dose	SOC	Published in PR journal	Duration of symptoms, recovery time to symptom free from enrollment, recovery time to symptom free from symptom onset, repeat PCR result on day 10
Raad 2021[113]	Lebanon	Double-blind	Self-funded	Asymptomatic outpatients	100	9 mg PO if 45 kg–64 kg, 12 mg PO if 65 kg–84 kg and 0.15 mg/kg if body weight ≥85 kg	Placebo	Prepublication data/ manuscript in progress obtained via email	Viral load reduction, hospitalization, adverse effects
Ravikirti 2021[109]	India	Double-blind	Self-funded	Mild to moderate COVID (inpatients)	112	12 mg × 2 days + SOC	Placebo + SOC	Published in PR journal	A negative RT-PCR report on day 6, symptomatic on day 6, discharge by day 10, admission to ICU, need for invasive mechanical ventilation, mortality
Rezai 2020[111]	Iran	Double-blind	None reported	Mild to moderate (inpatient)	60	0.2 mg/kg × 1 dose	SOC	Prepublication data obtained from another source	Clinical symptoms, respiratory rate and O2 saturation
Schwartz 2021[114,141]	Israel	Double-blind	None reported	Mild to moderate (outpatients)	94	0.15–0.3 mg/kg × 3 days	Placebo	Prepublication data obtained from another source	Viral clearance at day 4, 6, 8 and 10), hospitalization
COVID-19 prophylaxis studies									
Chahla 2021[142]	Argentina	Open-label	None reported	Health care workers	234	12 mg (in drops) weekly + iota-carrageenan 6 sprays daily × 4 wk	SOC	Prepublication data/ manuscript in progress obtained via email	COVID-19 infection (not clear if measured by PCR or symptoms)
Elgazzar 2020[47]	Egypt	Open-label	Self-funded	Health care and family contacts	200	0.4 mg/kg, weekly × 2 weeks	SOC	Research square preprint: emailed/ responded with data	Positive PCR test
Shouman 2020[143]	Egypt	Open-label	Self-funded	Family contacts	304	2 doses (15–24 mg depending on weight) on day 1 and day 3	SOC	Published in PR journal	Symptoms and/or positive COVID-19 PCR test within 14 days; adverse events

*Also administered doxycycline.
†multiarm trial.
SOC, standard of care; PR, peer review.

FOI 26-3132 - Document 3

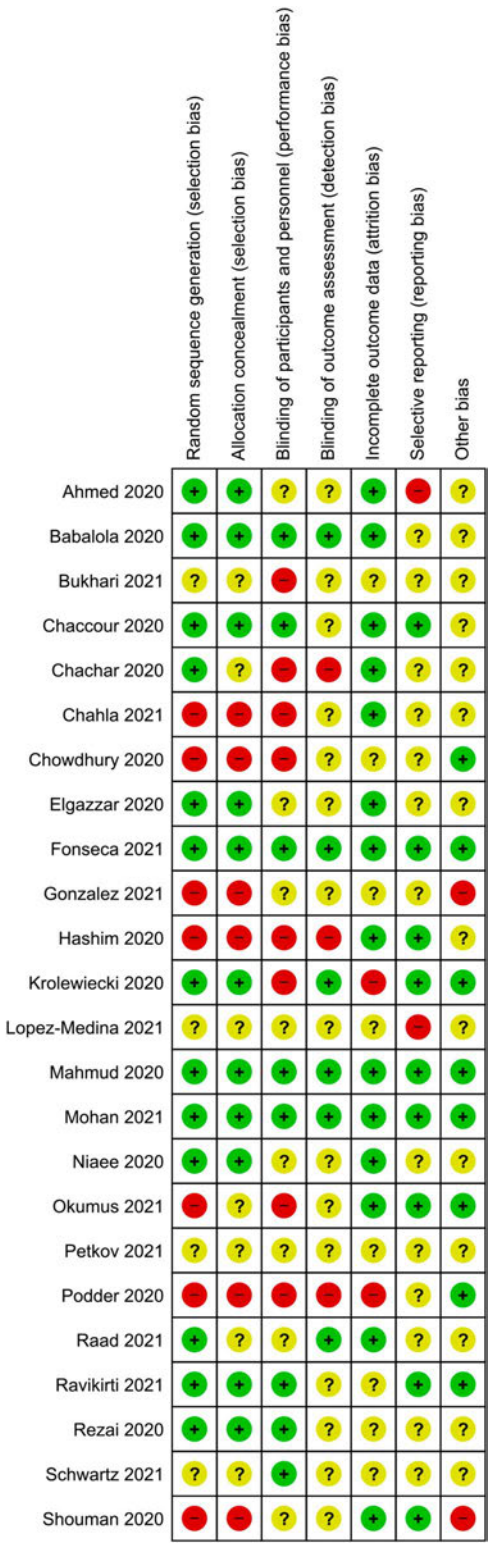


FIGURE 2. Risk-of-bias summary: review authors' judgments about each risk of bias item for each included study.

Ivermectin treatment versus no ivermectin treatment

Twenty-two trials (2668 participants) contributed data to the comparison ivermectin treatment versus no ivermectin treatment for COVID-19 treatment.

All-cause mortality

Meta-analysis of 15 trials, assessing 2438 participants, found that ivermectin reduced the risk of death by an average of 62% (95% CI 27%–81%) compared with no ivermectin treatment [average RR (aRR) 0.38, 95% CI 0.19 to 0.73; $I^2 = 49\%$]; risk of death 2.3% versus 7.8% among hospitalized patients in this analysis, respectively (SoF Table 2 and Figure 3). Much of the heterogeneity was explained by the exclusion of one trial[44] in a sensitivity analysis (average RR 0.31, 95% CI 0.17–0.58, n = 2196, $I^2 = 22\%$), but because this trial was at low risk of bias, it was retained in the main analysis. The source of heterogeneity may be due to the use of active comparators in the trial design. The results were also robust to sensitivity analyses excluding 2 other studies with an active treatment comparator (average RR 0.41, 95% CI 0.23–0.74, n = 1809, $I^2 = 8\%$). The results were also not sensitive to the exclusion of studies that were potentially at higher risk of bias (average RR 0.29, 95% CI 0.10–0.80, 12 studies, n = 2095, $I^2 = 61\%$), but in subgroup analysis, it was unclear as to whether a single dose would be sufficient. The effect on reducing deaths was consistent across mild to moderate and severe disease subgroups. Subgrouping data according to inpatient and outpatient trials was not informative because few outpatient studies reported this serious outcome. The conclusions of the primary outcome were also robust to a series of alternative post hoc analyses that explored the impact of numerous trials that reported no deaths in either arm. Extreme sensitivity analyses using a treatment arm continuity correction of between 0.01 and 0.5 did not change the certainty of the evidence judgments (Table 3).

Trial sequential analysis

TSA, using the DL random-effects method, showed that there may have been sufficient evidence accrued before the end of 2020 to show significant benefit of ivermectin over control for all-cause mortality. The cumulative z-curve in Figure 8 crossed the trial sequential monitoring boundaries after reaching the required IS, implying that there is firm evidence for a beneficial effect of ivermectin use over no ivermectin use in mainly hospitalized participants with mild to moderate COVID-19 infection.

Table 2. Summary of findings table of ivermectin versus no ivermectin for COVID-19 treatment in any setting.

Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	No. of participants (studies)	Quality of the evidence (GRADE)
	Assumed risk No ivermectin	Corresponding risk Ivermectin			
Death from any cause	78 per 1000 (all disease severity)	48 fewer deaths per 1000 (21–63)	RR = 0.38 (0.19–0.73)	2438 (15)	Moderate†
Recovery time to negative PCR test, in days	Absolute risks were not computed due to certainty of evidence being low and, in some cases, number of events being sparse		MD = −3.20 (−5.99 to −0.40)	375 (6)	Very low†,‡,§
Time to clinical recovery, in days (outpatients)			MD = −1.06 (−1.63 to −0.49)	176 (2)	Very low†,‡,§
Time to clinical recovery, in days (mild to moderate COVID-19 inpatients)			MD = −7.32 (−9.25 to −5.39)	96 (1)	Very low†,¶
Time to clinical recovery, in days (severe COVID-19 inpatients)			MD = −3.98 (−10.06 to 2.10)	33 (1)	Very low†,¶
Admission to ICU			RR=1.22 (0.75–2.00)	379 (2)	Very low¶,‖
Need for mechanical ventilation			RR=0.66 (0.14–3.00)	431 (3)	Low§,‖
Length of hospital stay, in days			MD= 0.13 (−2.04 to 2.30)	68 (1)	Very low†,¶
Admission to hospital			RR 0.16 (0.02–1.32)	194 (2)	Very low†,¶
Duration of mechanical ventilation	Not reported				
Improvement (mild to moderate COVID-19)*	635 improved per 1000	159 more per 1000 (from 51 more to 286 more)	RR 1.25 (1.08–1.45)	681 (5)	Low†,‡
Deterioration (any disease severity)	143 per 1000	93 fewer per 1000 (from 50 fewer to 116 fewer)	RR 0.35 (0.19–0.65)	1587 (7)	Low†,‡
Serious adverse events	7/867 (0.8%) had an SAE in ivermectin group and 2/666 (0.3%) in control		RR=1.65 (0.44–6.09)	1533 (11)	Low†,‡

*Only one study contributed to the "severe" COVID-19 subgroup and subgroup data were not pooled due to subgroup differences.
†Downgraded −1 for study design limitations.
‡Downgraded −1 for inconsistency.
§Downgraded −1 for imprecision.
¶Downgraded −2 for imprecision/sparse data.
‖Downgraded −1 for indirectness.

The TSA was used to calculate the IS required to demonstrate or reject a 62% RRR of death in the ivermectin group, as observed in the primary meta-analysis. This estimate is similar to effect estimates reported in other reviews.[10] We assumed a 7.8% event proportion in the control group, which was the average control group

FOI 26-3132 - Document 3

Table 3. Sensitivity analyses for death from any cause considering methods for dealing with zero events in trials.

Method	Measure	Model	Effect size (95% CI)	Details
Peto	OR	FE	0.35 (0.24 to 0.53)	Handles single-zero trials
M-H	OR	FE	0.37 (0.24 to 0.56)	Handles single-zero trials
M-H	OR	RE	0.33 (0.16 to 0.68)	Handles single-zero trials
M-H	RR	FE	0.42 (0.29 to 0.60)	Handles single-zero trials
M-H	RR	RE	0.37 (0.19 to 0.74)	Handles single-zero trials
M-H	RD	FE	−0.04 (−0.06 to −0.02)	Handles double-zero trials
M-H	RD	RE	−0.03 (−0.06 to −0.00)	Handles double-zero trials
IV	RD	FE	−0.01 (−0.02 to −0.00)	Handles double-zero trials
IV	RD	RE	−0.02 (−0.04 to −0.00)	Handles double-zero trials
Treatment arm continuity correction methods using IV			Accounting for double zeros	Accounting for all zeros
0.01	RR	FE	0.54 (0.36 to 0.79)	0.58 (0.39–0.88)
0.01	RR	RE	0.43 (0.25 to 0.72)	0.58 (0.39–0.88)
0.1	RR	FE	0.54 (0.37 to 0.79)	0.56 (0.38–0.84)
0.1	RR	RE	0.43 (0.26 to 0.73)	0.46 (0.26–0.80)
0.25	RR	FE	0.54 (0.37 to 0.79)	0.55 (0.37–0.81)
0.25	RR	RE	0.44 (0.26 to 0.73)	0.45 (0.26–0.76)
0.5	RR	FE	0.54 (0.37 to 0.79)	0.55 (0.35–0.78)
0.5	RR	RE	0.45 (0.27 to 0.74)	0.47 (0.29–0.75)

FE, fixed effects; IV, inverse variance; M-H, Mantel-Haenszel; RD, risk difference; RE, random effects; TACC, treatment arm continuity correction.

event rate from the primary meta-analysis. We used a model variance-based estimate of 49.1% (diversity estimate) to correct for heterogeneity. The required IS was 1810 participants (Figure 8), which was exceeded by the total number of observed participants in the meta-analysis (n = 2438). In the TSA plots, the red dashed lines in Figure 8 represent the trial sequential monitoring boundaries using the O'Brien–Fleming alpha-spending function. The solid blue line is the cumulative z-curve and represents the observed trials in the cumulative meta-analysis. The adjusted significance boundaries for the cumulative z-curve were constructed under the assumption that significance testing may have been performed each time a new trial was added to the meta-analysis. In Figure 8, the z-curve crosses the boundary after reaching the required IS, thereby supporting the previous conclusion in RevMan 5.4.1[31] using the DL method that ivermectin is superior to control in reducing the risk of death.

Sensitivity analyses

Sensitivity analysis excluding the trial of Fonseca[44] significantly reduced heterogeneity in the meta-analysis and thus the diversity estimate in the TSA using the DL model. This strengthened the suggestion in the primary core analysis that the required IS had been reached (Figure 9). Because the DL estimator could potentially underestimate the between-trials variance,[43] we performed further sensitivity analyses using 2 alternative random-effects model approaches. The results of the primary TSA analysis were robust to sensitivity analysis using the BT method with the same parameters, excluding the Fonseca[44] trial, which was a cause of substantial heterogeneity (Figure 10). The TSA

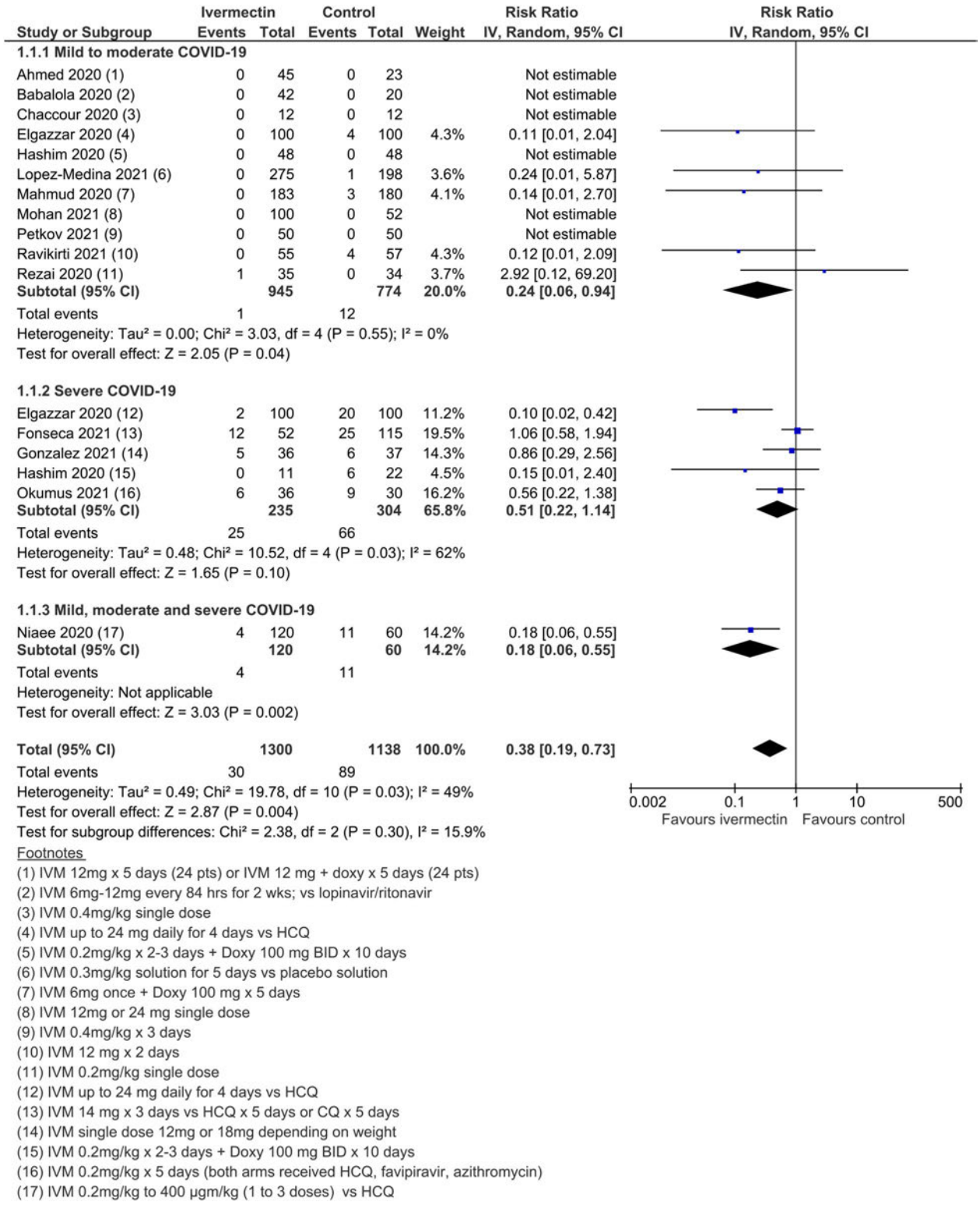


FIGURE 3. Death due to any cause.

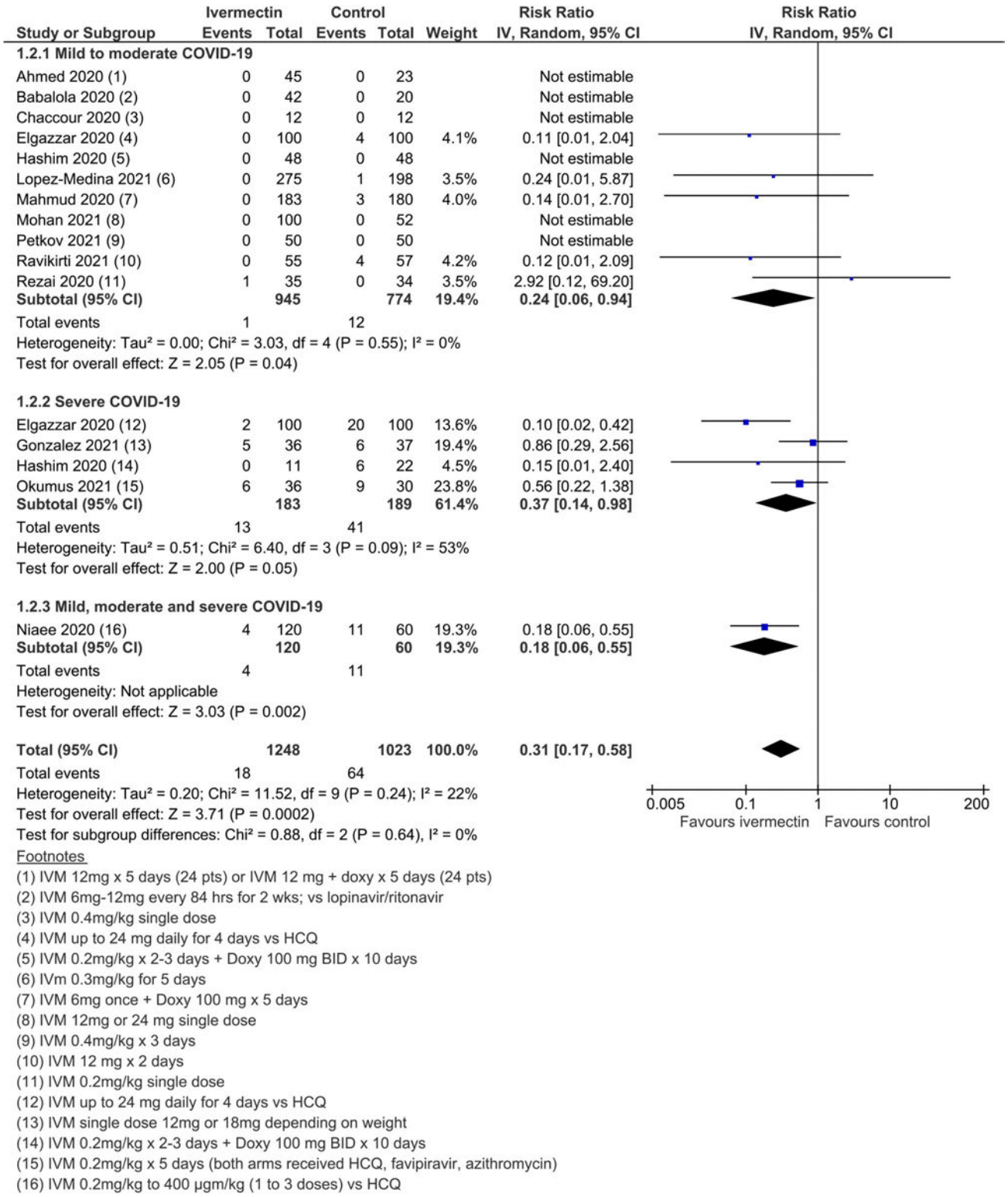


FIGURE 4. Death due to any cause, excluding an outlier study responsible for the heterogeneity.

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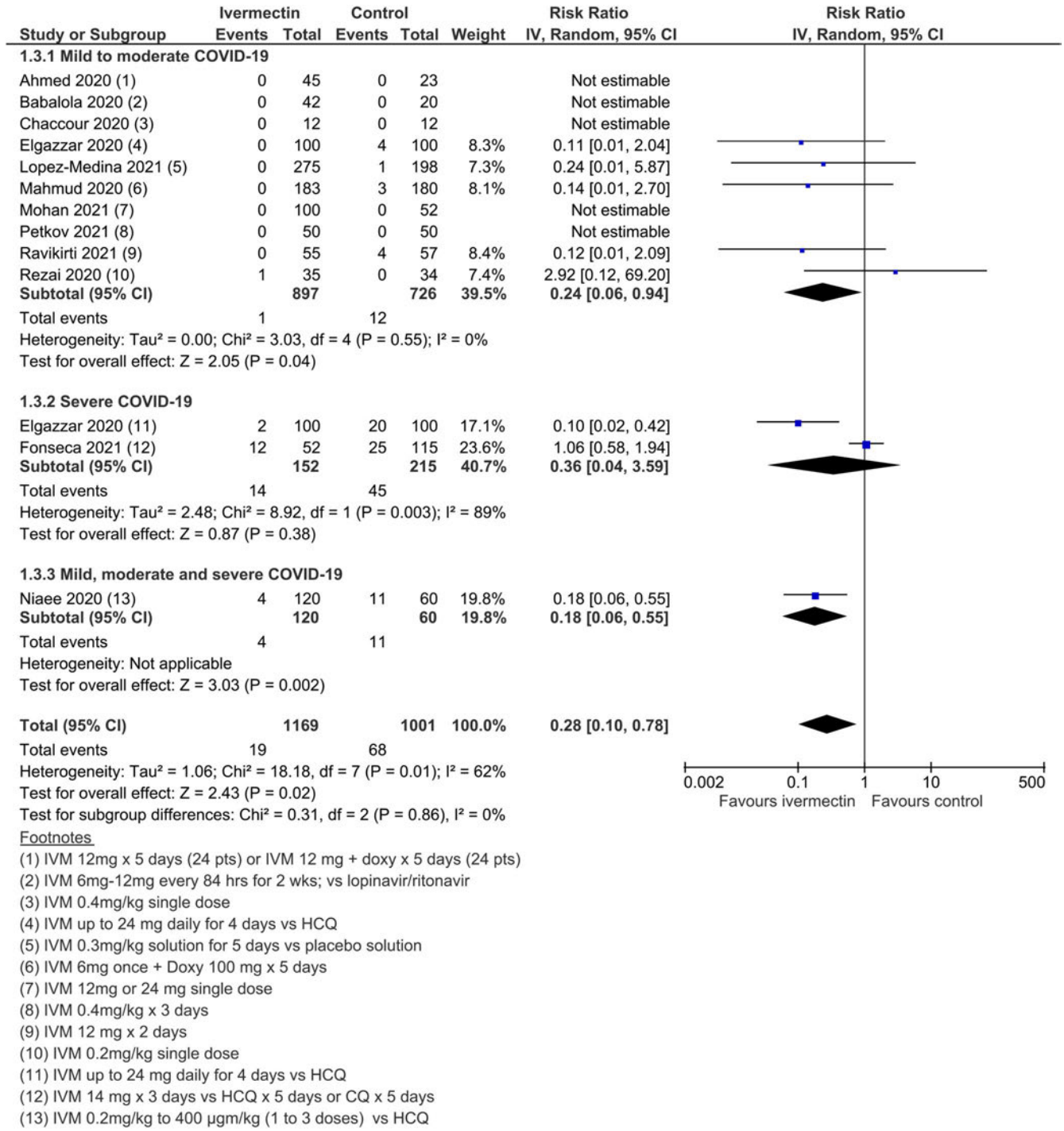


FIGURE 5. Death due to any cause, excluding high risk-of-bias studies.

comprehensively confirms the result of the conventional meta-analysis. The required IS was 1064.

The required IS was not reached in the TSA using the SJ method, largely because diversity from the model was high (Figure 11). The SJ estimator may overestimate the between-trials variance in meta-analyses with mild heterogeneity, thus producing artificially wide confidence intervals.[43] When the diversity estimate was reduced to the same as in the DL model, the required IS was reached in the SJ model (data not shown). There was no evidence of futility using the SJ method in any scenario.

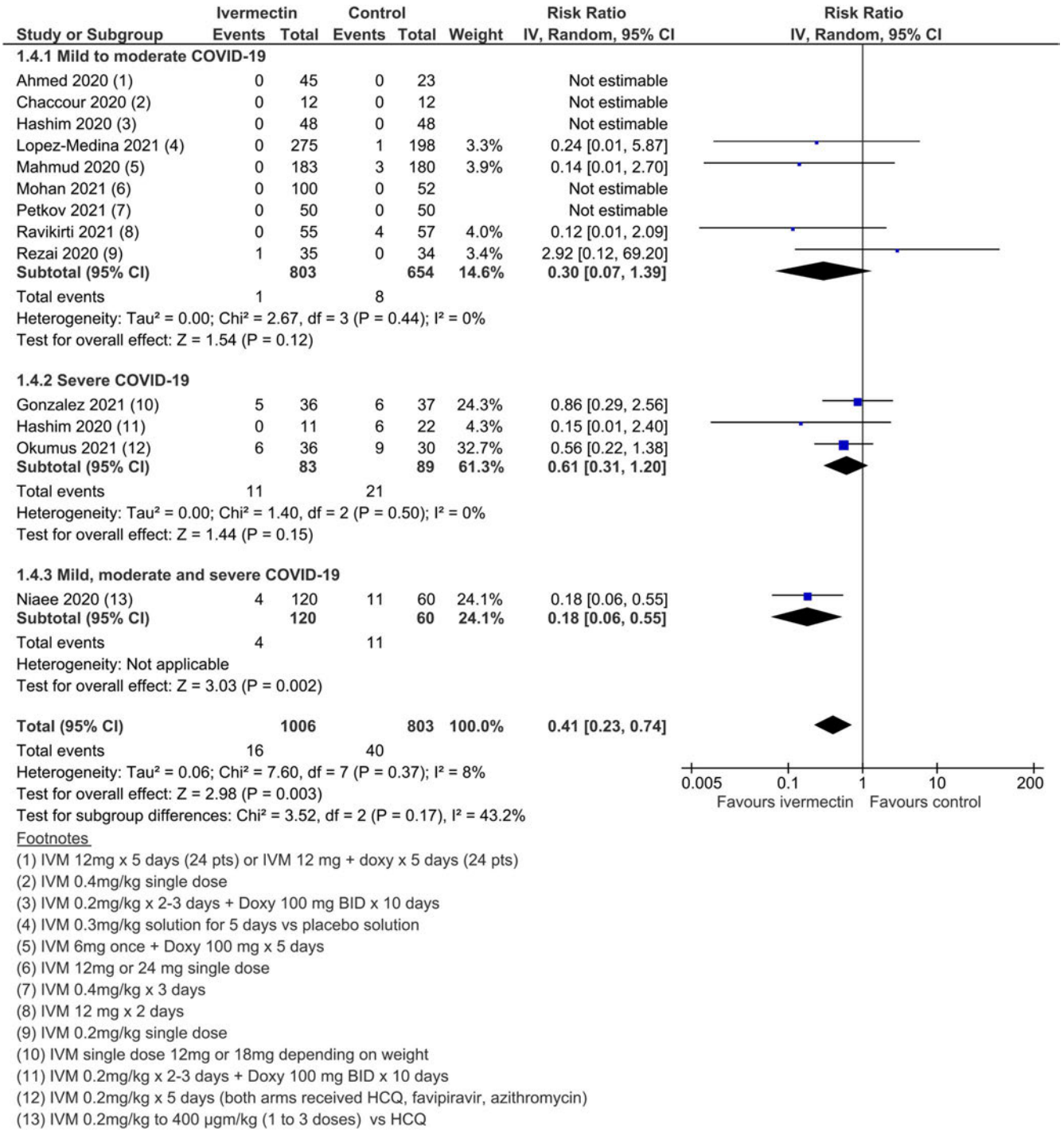


FIGURE 6. Death due to any cause, excluding studies with active controls.

Certainty of the evidence for all-cause mortality

Overall, death from any cause, taking into account all composite analyses, was judged to provide moderate-certainty evidence (SoF Table 2 and Figures 4–11). A funnel plot corresponding to the primary outcome of death from any cause did not seem to suggest any evidence of publication bias (Figure 7). Furthermore, the ease with which trial reports can be uploaded as preprints should reduce this risk.

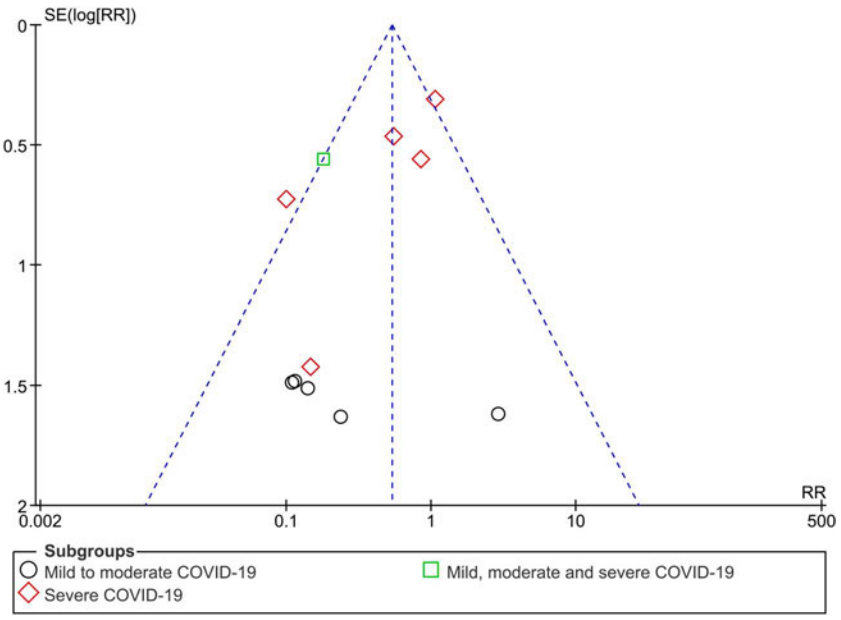


FIGURE 7. Funnel plot of ivermectin versus control for COVID-19 treatment for all-cause death (subgrouped by severity).

Secondary outcomes

Secondary outcomes provided low to very low certainty evidence (SoF Table 2). Low-certainty findings suggested that there may be no benefit with ivermectin for "need for mechanical ventilation," whereas effect estimates for "improvement" and "deterioration" favored ivermectin but were graded as low certainty due to study design limitations and inconsistency (Figures 12–14). All other secondary outcome findings were assessed as very low certainty.

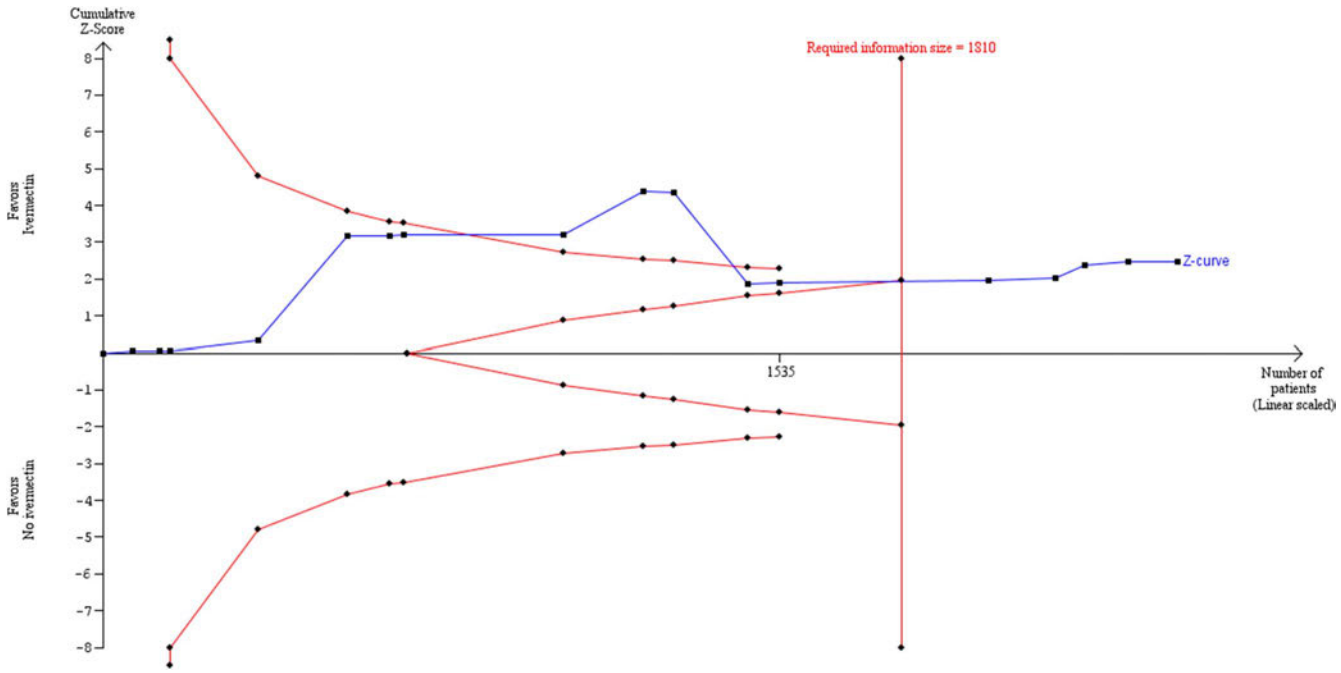


FIGURE 8. Trial sequential analysis using DL random-effects method with parameter estimates of $\alpha = 0.05$, $\beta = 0.1$, control rate = 7.8%, RRR = 62%, and diversity = 49.5%.

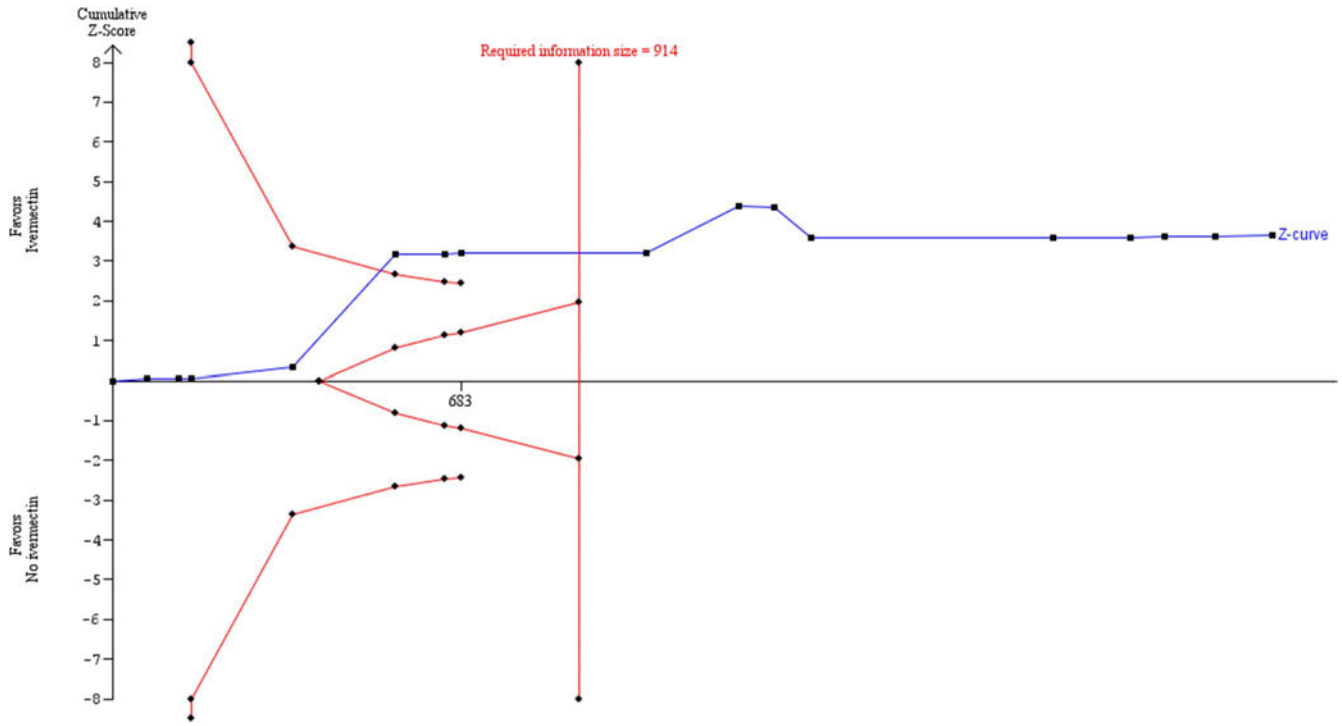


FIGURE 9. Sensitivity analysis excluding an outlier study responsible for the heterogeneity, showing trial sequential analysis using DL random-effects method with parameter estimates of $\alpha = 0.05$, $\beta = 0.1$, control rate = 7.8%, = 62%, and diversity = 0%.

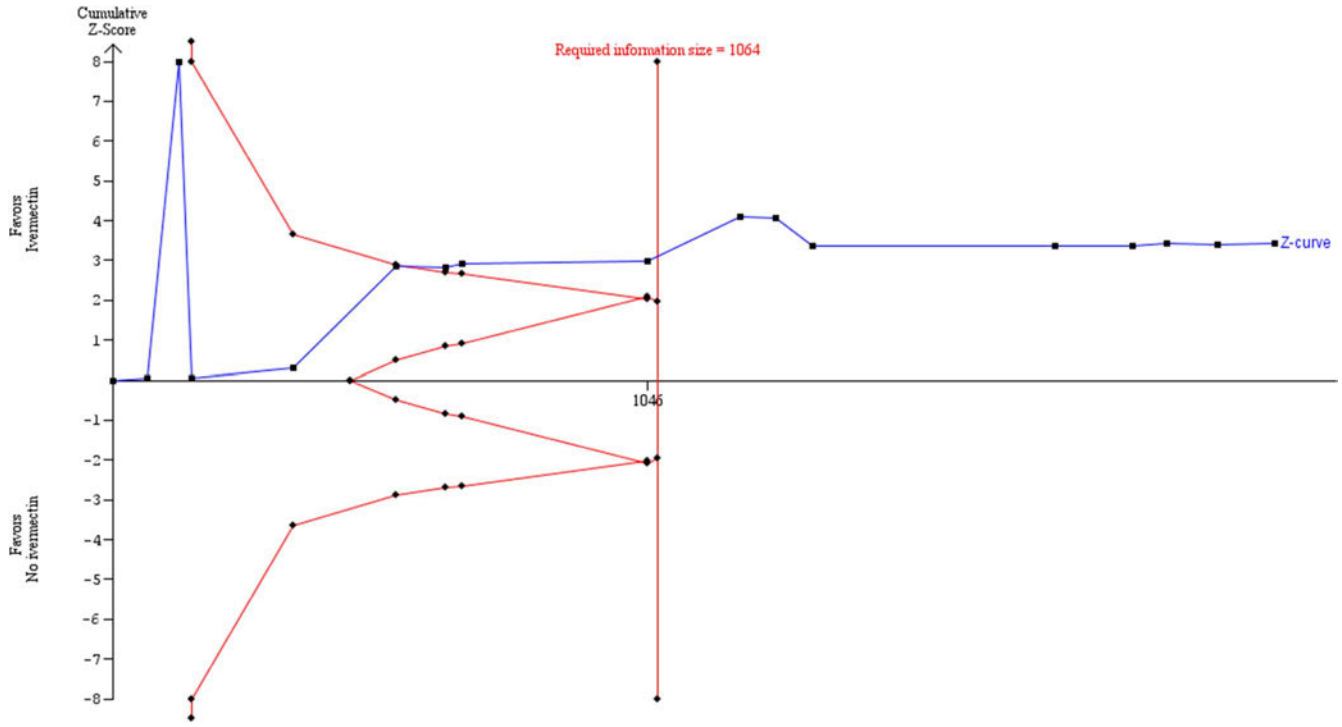


FIGURE 10. Sensitivity analysis excluding an outlier study responsible for the heterogeneity, showing trial sequential analysis using Biggerstaff–Tweedie random-effects method with parameter estimates of $\alpha = 0.05$, $\beta = 0.1$, control rate = 7.8%, RRR = 62%, and diversity = 14.2%.

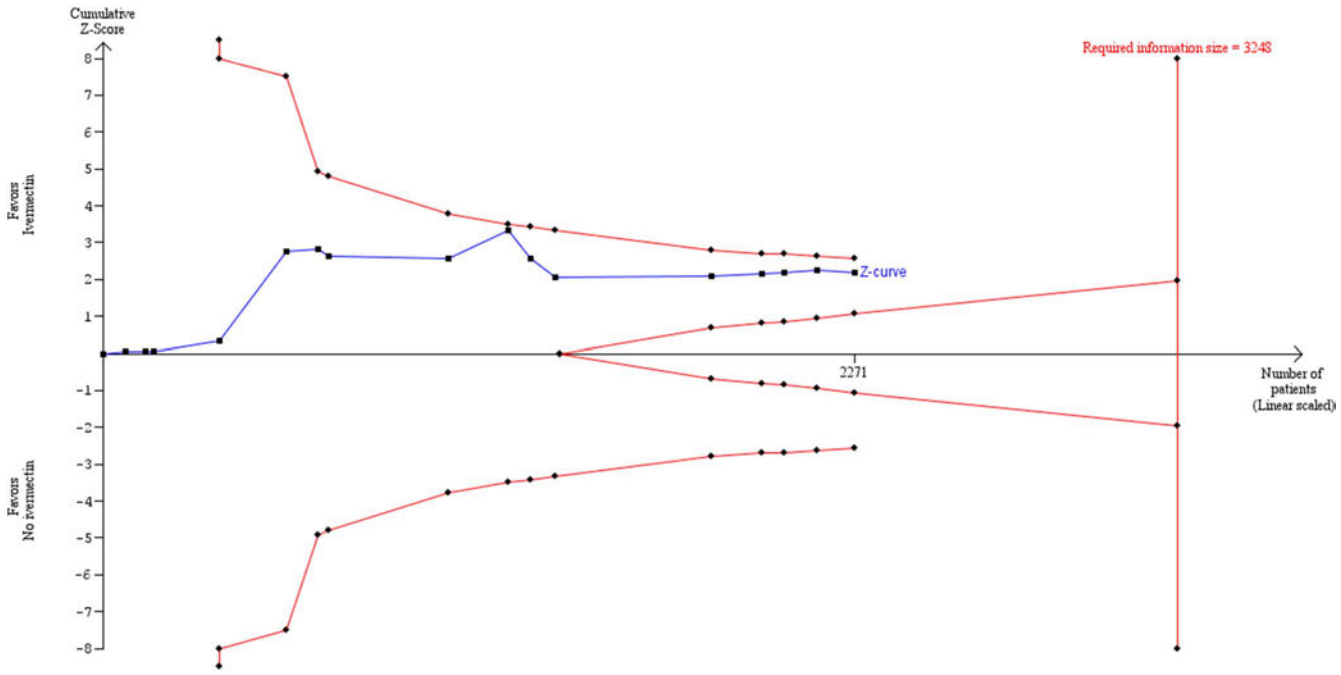


FIGURE 11. Sensitivity analysis excluding an outlier study responsible for the heterogeneity, showing trial sequential analysis using Sidik–Jonkman random-effects method with parameter estimates of $\alpha = 0.05$, $\beta = 0.1$, control rate = 7.8%, RRR = 62%, and diversity = 71.9%.

Meta-analysis of 11 trials, assessing 1533 participants, found that there was no significant difference between ivermectin and control in the risk of severe adverse events (aRR 1.65, 95% CI 0.44–6.09; $I^2 = 0\%$; *low certainty evidence*, downgraded for imprecision and study design limitations). Seven severe adverse events were reported in the ivermectin group and 2 in controls. The SAEs were as follows: 2 patients in the Mahmud trial[107] had esophagitis (this is a known side effect of doxycycline, which was coadministered with ivermectin in this trial); one patient in the study by Krolewiecki et al[106] had hyponatremia (this trial used high-dose ivermectin for 5 days); and 2 patients in a study from Turkey[115] had serious "delirium-like behavior, agitation, aggressive attitude, and altered state of consciousness," which the authors attributed to metabolic insufficiencies in MDR-1/ABCB1 or CYP3A4 genes, screening for which was a study feature. In the Lopez-Medina et al[85] trial, there were 2 SAEs in each arm (SoF Table 2).

Ivermectin prophylaxis versus no ivermectin prophylaxis

Three studies involving 738 participants evaluated ivermectin for COVID-19 prophylaxis among health care workers and COVID-19 contacts. Meta-analysis of these 3 trials, assessing 738 participants, found that ivermectin prophylaxis among health care workers and COVID-19 contacts probably reduces the risk of

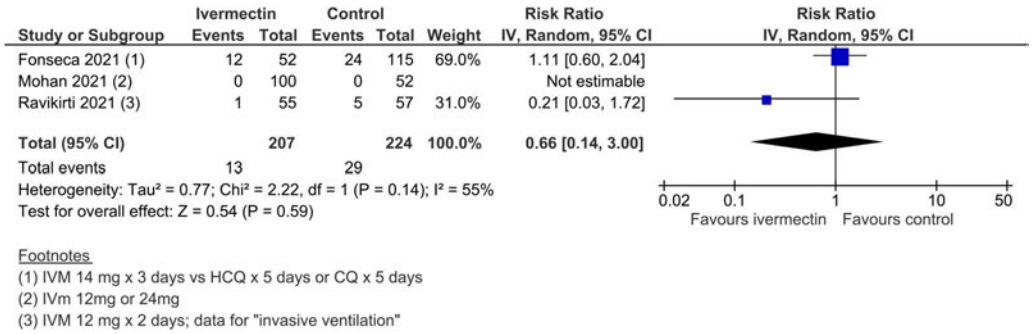
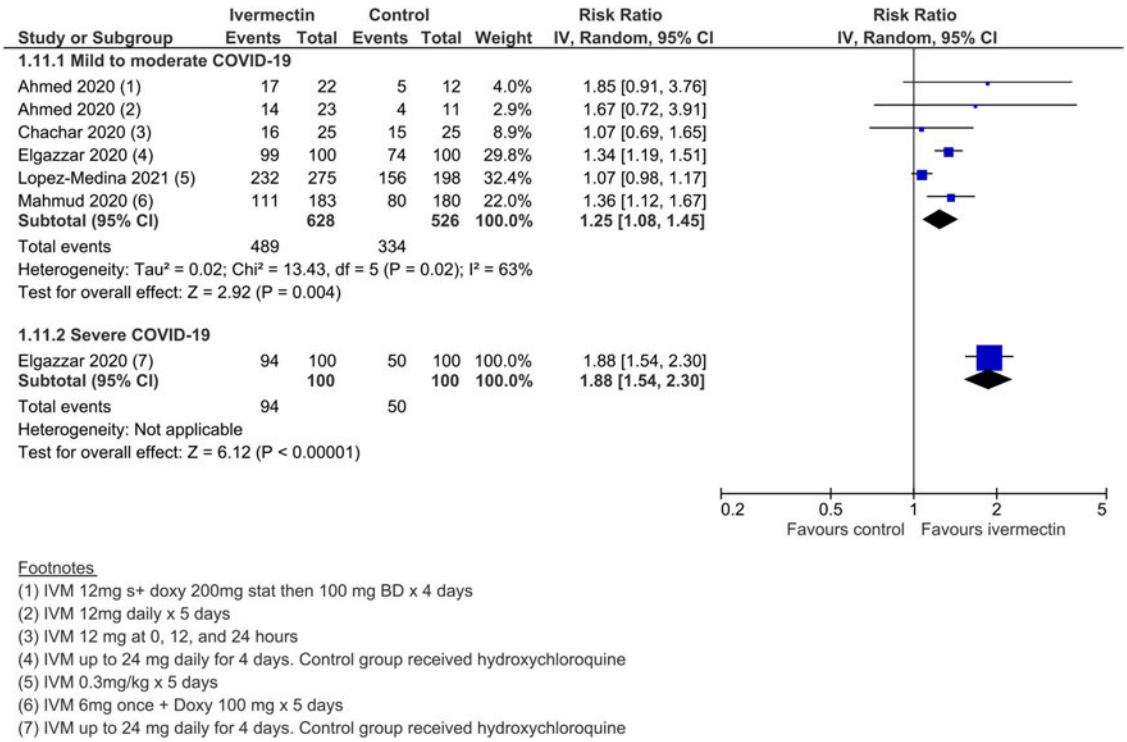


FIGURE 12. Need for mechanical ventilation.



Footnotes
(1) IVM 12mg s+ doxy 200mg stat then 100 mg BD x 4 days
(2) IVM 12mg daily x 5 days
(3) IVM 12 mg at 0, 12, and 24 hours
(4) IVM up to 24 mg daily for 4 days. Control group received hydroxychloroquine
(5) IVM 0.3mg/kg x 5 days
(6) IVM 6mg once + Doxy 100 mg x 5 days
(7) IVM up to 24 mg daily for 4 days. Control group received hydroxychloroquine

FIGURE 13. Improvement.

COVID-19 infection by an average of 86% (79%–91%) (3 trials, 738 participants; aRR 0.14, 95% CI 0.09–0.21; 5.0% vs. 29.6% contracted COVID-19, respectively; *low-certainty evidence*; downgraded due to study design limitations and few included trials) (Figure 15). In 2 trials involving 538 participants, no severe adverse events were recorded (SoF Table 4).

DISCUSSION

The findings indicate with moderate certainty that ivermectin treatment in COVID-19 provides a significant survival benefit. Our certainty of evidence judgment was consolidated by the results of trial sequential analyses, which show that the required IS has probably already been met. Low-certainty evidence on improvement and deterioration also support a likely clinical benefit of ivermectin. Low-certainty evidence suggests a significant effect in prophylaxis. Overall, the evidence also suggests that early use of ivermectin may reduce morbidity and mortality from COVID-19. This is based on (1) reductions in COVID-19 infections when ivermectin was used as prophylaxis, (2) the more favorable effect estimates for mild to moderate disease compared with severe disease for death due to any cause, and (3) on the evidence demonstrating reductions in deterioration.

The evidence on severe adverse events in this review was graded as low certainty, partly because there were too few events to reach statistical significance. Evidence from a recent systematic review of ivermectin use among people with parasitic infections suggests that ivermectin administered at the usual doses (0.2 or 0.4 mg/kg) is safe and could be safe at higher doses.[7,116] A recent World Health Organization document on ivermectin use for scabies found that adverse events with ivermectin were primarily minor and transient.[22]

We restricted the included studies to the highest level of evidence, that is, RCTs, as a policy. This was despite there being numerous observational but nonrandomized trials of ivermectin, which one could argue could also be considered in an emergency. We included preprint and unpublished data from completed but not yet published trials due to the urgency related to evidence synthesis in the context of a global pandemic.[117] Although there is the potential for selective reporting of outcomes and publication bias, we have factored in these considerations in interpreting results and forming conclusions. We adhered to PRISMA guidelines and the WHO statement on developing global norms for sharing data and results during public health emergencies.[117]

There are a number of limitations with this review. Several of the studies contributing data did not

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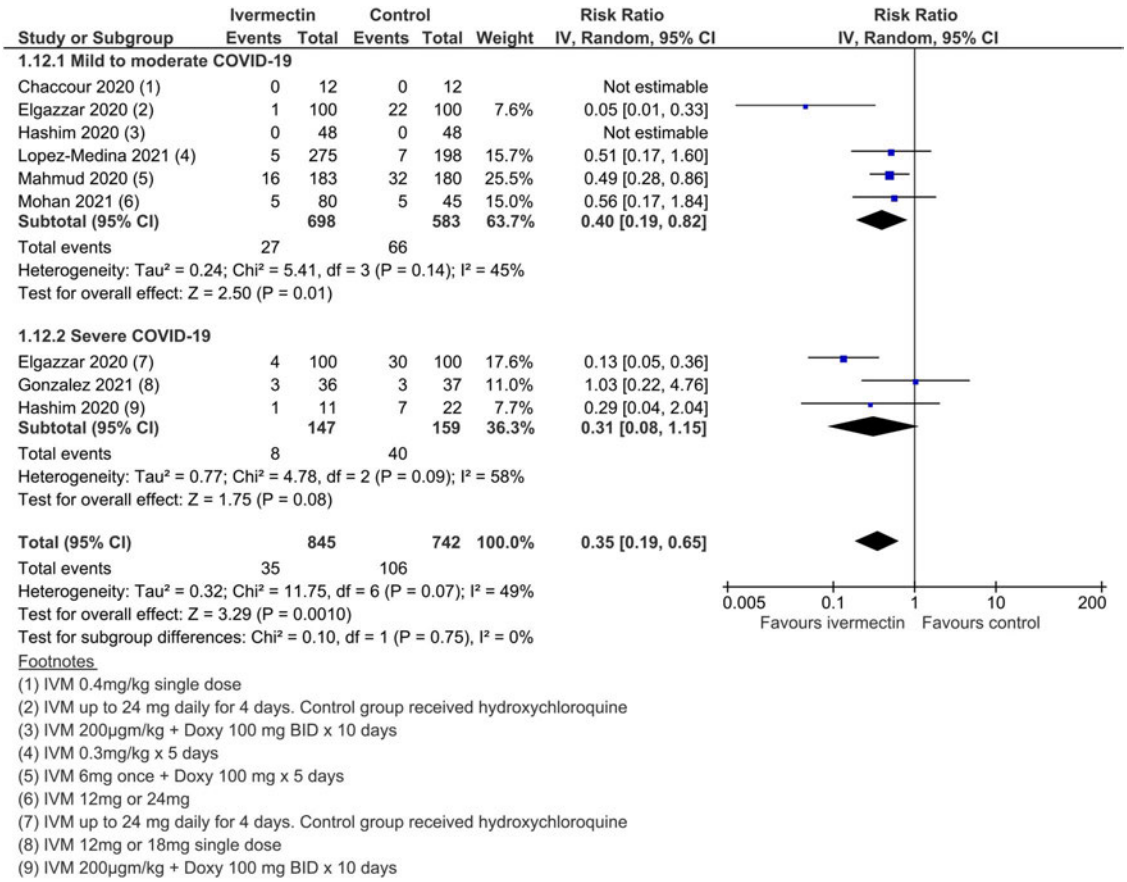


FIGURE 14. Deterioration.

provide full descriptions of methods, so assessing risk of bias was challenging. Where descriptions of study methods were sparse or unclear, we attempted to contact authors to clarify methods, but lack of information led us to downgrade findings in several instances. Overall interpretation of findings was hampered due to variability in the participants recruited, treatment regimen, and the care offered to those in control groups. We have tried to take this variation into account through subgroup and sensitivity analyses. Nevertheless, dosing and treatment regimens and the use of ivermectin with other components of "standard care" require further research. We did not include laboratory outcome measures, such as viral clearance. The latter and other biochemical outcomes have been reported in several studies and reviews and tend to favor ivermectin.[10,47,105,108] Several trials reported continuous data, such as length of hospital stay, as medians and interquartile ranges; therefore, we were unable to include these data in meta-analysis. Because we did not undertake in our protocol to perform narrative evidence synthesis, and because these data tended to favor ivermectin, the certainty of the effects of ivermectin on these continuous outcomes may be underestimated.

At least 5 other reviews of ivermectin use for COVID-19 have been published, including one coauthored with Nobel Laureate Professor Satoshi Ōmura, discoverer of ivermectin,[9,10,118,119,120] but only 3 have been peer-reviewed[9,118,120] and only 2 attempt full systematic review.[10,119] We applied AMSTAR 2,[121] a critical appraisal tool for systematic reviews of health care interventions, to the 2 nonpeered systematic reviews[10,119] and both were judged to be of low quality (Table 5). However, there was also a suggestion that ivermectin reduced the risk of death in treatment of COVID-19 in these reviews.

The recently updated WHO therapeutics guidelines[12] included 7 trials and 1419 people in the analysis of mortality. Reporting a risk reduction of 81% (odds ratio 0.19, 95% CI 0.09–0.36), the effect estimate favoring ivermectin was downgraded by 2 levels for imprecision, although the justification for this is unclear as the reported CI is precise (64%–91%).

In addition to the evidence from systematic reviews, the findings of several controlled observational studies

FOI 26-3132 - Document 3

Table 4. Summary of findings table of ivermectin versus no ivermectin for COVID-19 prophylaxis in healthy population (people without COVID-19 infection).

	Illustrative comparative risks* (95% CI)				
	Assumed risk	Corresponding risk			
Outcomes	No ivermectin	Ivermectin	Relative effect (95% CI)	No of participants (studies)	Quality of the evidence (GRADE)
COVID-19 infection	296 per 1000	245 fewer infections per 1000 (234–269)	RR = 0.14 (0.09–0.21)	738 (3)	Low†
Admission to hospital	Not reported				
Death from any cause	Not reported				
Serious adverse events	No events occurred in 538 participants (2 studies), therefore the effect could not be estimated.				

GRADE working group grades of evidence; High quality: Further research is very unlikely to change our confidence in the estimate of effect; Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate; Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate; Very low quality: We are very uncertain about the estimate.
*The basis for the assumed risk (eg, the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% CI) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).
†Downgraded −2 for study design limitations.
NNT, number needed to treat.

are consistent with existing evidence and suggest improved outcomes with ivermectin treatment.[55,57,59] Similarly, with respect to ivermectin prophylaxis of frontline workers and those at risk, controlled observational studies from Bangladesh and Argentina (the latter which involved 1195 health care workers) have shown apparent reductions in COVID-19 transmission with ivermectin prophylaxis, including in some reports total protection (zero infections) where infection rates in the control group exceeded 50%.[122,123] A very large trial of ivermectin prophylaxis in health care workers in India[124] covered 3532 participants and reported risk ratios not significantly different from this meta-analysis (prophylaxis outcome).

Clarifying ivermectin safety in pregnancy is a key question in patient acceptability for pregnant women contracting COVID-19. A recent meta-analysis[5] found little evidence of increased risk of abnormal pregnancies but similarly weak evidence of absence of risk. For (pre-exposure) prophylaxis in pregnancy, where vaccines may be contraindicated, the alternative of hydroxychloroquine has been advocated.[125,126] In addition to safety and relative efficacy, different risk–benefit judgments may be presented for prophylaxis

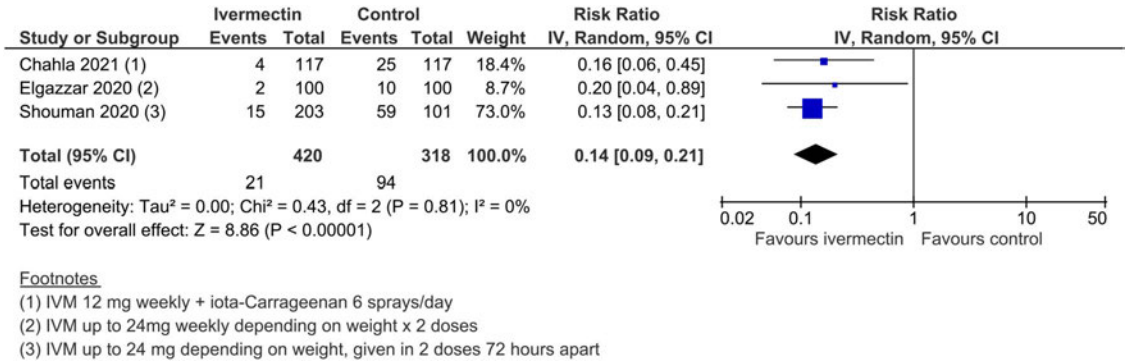


FIGURE 15. COVID-19 infection (prophylaxis studies).

Table 5. Methodological quality of other systematic reviews (AMSTAR 2).

Systematic review	Components of PICO described	A priori study design	Explain selection of study designs	Comprehensive literature search	Duplicate study selection	Duplicate data extraction	List of excluded studies justified	Characteristics of included studies provided
Hill et al, 2021[10]	+	−	+	+	?	?	−*	?†
Castañeda-Sabogal et al 2021[119]	+‖	?	−	?#	+	+	−*	+

Systematic review	Risk of bias adequately assessed and documented	Sources of funding reported	Appropriate methods to combine findings	Appropriate risk-of-bias sensitivity analyses conducted	Risk-of-bias assessment used in conclusions	Satisfactory explanation of observed heterogeneity	Likelihood of publication bias assessed	Conflict of interest stated
Hill et al, 2021[10]	−‡	−	−§	−*	−¶	−*	NA	−
Castañeda-Sabogal et al 2021[119]	−**	−	−††	−‡‡	−*	+	NA	+

Assessed using AMSTAR 2[121]; +, adequately assessed; −, inadequately assessed; ?, unclear assessment; NA, not applicable (less than 10 included studies in meta-analysis).

*Not documented or inadequately reported.

†Participant population, description of comparator interventions, and time frame for follow-up were not described or inadequately reported.

‡No summary of risk-of-bias assessment was given in the main text in the review, other than stating trials were of poor, fair, or high quality. There were some further details about bias in the discussion, but these were largely generic and did not follow the recommended Cochrane tool used to assess risk of bias in RCTs.

§A meta-analysis for all-cause death was presented but authors did not specify why meta-analyses were not conducted for other outcomes, which included at least 2 trials reporting the same comparison and outcome, other than in some parts of the discussion. For example, if viral clearance was reported in most trials, there would have been scope to have performed subgroup analyses and/or split the time point for each comparison to account for the varying duration of follow-up across trials. Instead, they gave a vote count-type narrative of the results, which did not follow synthesis without meta-analysis (SWiM) in systematic review reporting guidelines.[144]

¶There was some further details about bias in the discussion, but this was largely generic and did not follow the recommended Cochrane tool used to assess risk of bias in RCTs. Similarly, in terms of certainty/quality of the evidence, the authors used terms in a summary table that included "good," "fair," and "limited," without offering any explanation or justification.

‖Outcomes were reported but lacked definitions.

#A significant number of pertinent RCTs have not been included in the review. Given the adequate due diligence of review process, the comprehensive nature of the search strategy is questionable.

**No description of risk-of-bias assessment in any domain apart from missing outcome data but attrition rates not documented to justify judgment.

††Authors did not report data from RCTs that we obtained from various sources and some conclusions were not reflective of the observed data. It was reported that in an analysis of 4 preprint retrospective studies at high risk of bias, ivermectin was not associated with reduced mortality (logRR 0.89, 95% CI 0.09–1.70, $P = 0.04$). Although the caveat of studies being at high risk of bias and statistical heterogeneity should be added to any interpretation, it is incorrect to interpret these results as not demonstrating a potential association based on the observed result. Furthermore, the high risk of bias judgment is not adequately justified.

‡‡A sensitivity analysis was performed excluding those studies without adjustment for confounding but no details are provided. Given that there was some evidence of a potential association with ivermectin treatment and survival in 4 retrospective studies (although downplayed as no association due to concerns about attrition), it is highly implausible that any sensitivity analysis would not remove any suggestion of association.

FOI 26-3132 - Document 3

(pre- and post-exposure), and for treatment, with pregnancy a high-risk status for COVID-19.

RCTs in this review did not specifically examine use of ivermectin in the elderly, although this is a known high-risk group for severe COVID-19. In the setting of care homes, it is also notorious for rapid contagion. A standard indication for ivermectin in the elderly is scabies. We identified 2 recent reports suggesting that ivermectin may be efficacious as prevention and treatment of COVID-19 in this age group.[50,127] A letter on positive experience in 7 elder care facilities in Virginia covering 309 patients was sent to NIH[127] and has recently been submitted for publication.

There is also evidence emerging from countries where ivermectin has been implemented. For example, Peru had a very high death toll from COVID-19 early on in the pandemic.[128] Based on observational evidence, the Peruvian government approved ivermectin for use against COVID-19 in May 2020.[128] After implementation, death rates in 8 states were reduced between 64% and 91% over a two-month period.[128] Another analysis of Peruvian data from 24 states with early ivermectin deployment has reported a drop in excess deaths of 59% at 30+ days and of 75% at 45+ days.[129] However, factors such as change in behavior, social distancing, and face-mask use could have played a role in this reduction.

Other considerations related to the use of ivermectin treatment in the COVID-19 pandemic include people's values and preferences, equity implications, acceptability, and feasibility.[130] None of the identified reviews specifically discussed these criteria in relation to ivermectin. However, in health care decision making, evidence on effectiveness is seldom taken in isolation without considering these factors. Ultimately, if ivermectin is to be more widespread in its implementation, then some considerations are needed related to these decision-making criteria specified in the GRADE-DECIDE framework.[130]

There are numerous emerging ongoing clinical trials assessing ivermectin for COVID-19. The trade-off with policy and potential implementation based on evidence synthesis reviews and/or RCTs will vary considerably from country to country. Certain South American countries, Indian states, and, more recently, Slovakia and other countries in Europe have implemented its use for COVID-19.[129,131,132,133,134] A recent survey of global trends[118] documents usage worldwide. Despite ivermectin being a low-cost medication in many countries globally, the apparent shortage of economic evaluations indicates that economic evidence on ivermectin for treatment and prophylaxis of SARS-CoV-2 is currently lacking. This may impact more on LMICs that are potentially waiting for guidance from organizations like the WHO.

Given the evidence of efficacy, safety, low cost, and current death rates, ivermectin is likely to have an impact on health and economic outcomes of the pandemic across many countries. Ivermectin is not a new and experimental drug with an unknown safety profile. It is a WHO "Essential Medicine" already used in several different indications, in colossal cumulative volumes. Corticosteroids have become an accepted standard of care in COVID-19, based on a single RCT of dexamethasone.[1] If a single RCT is sufficient for the adoption of dexamethasone, then a fortiori the evidence of 2 dozen RCTs supports the adoption of ivermectin.

Ivermectin is likely to be an equitable, acceptable, and feasible global intervention against COVID-19. Health professionals should strongly consider its use, in both treatment and prophylaxis.

ACKNOWLEDGMENTS

This work was inspired by the prior literature review of Dr Pierre Kory.

The authors thank Information Specialist, Jo Platt, of the Cochrane Gynaecological, Neuro-oncology, and Orphan Cancer (CGNOC) group for designing the search strategy and running the search, as well as Anna Noel Storr for reviewing the strategy. The authors also thank Isabella Rushforth for her voluntary assistance with the preparation of the reference lists.

The authors thank Gill Gyte for detailed comments, feedback, and involvement in this review, and Michael Grayling and David Tovey for useful peer review comments before submission. The authors also thank the external peer reviewers for their helpful comments and Peter Manu for the opportunity to publish our findings.

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FOI 26-3132 - Document 3

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Effectiveness of Ivermectin-Based Multidrug Therapy in Severe Hypoxic Ambulatory COVID-19 Patients

Running head: Ivermectin combo therapy treats COVID-19 patients

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Acknowledgements: The authors would like to thank Margaux Alvaran for technical assistance.

Funding: This study was funded by ProgenaBiome, LLC.

The authors state that they have obtained appropriate institutional review board approval and have followed principles of the Declaration of Helsinki for all human experimental investigations. Informed consent has been obtained from the participants involved.

Data-sharing: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Word count (intro+methods+results+discussion): 3576
Figure number: 3+ 1 Supplementary figure
Table number: 2

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Abstract

Ivermectin is a safe, inexpensive and effective early COVID-19 treatment validated in 20+ RCTs. Having developed combination therapies for *Helicobacter pylori*, we tested various COVID-19 combinations and describe the most effective. In 24 consecutive COVID-19 subjects with high risk features, hypoxia and untreated moderate-severe symptoms averaging 9 days, we trialed this novel combination comprising ivermectin, doxycycline, zinc, and Vitamins D and C. It was highly effective. All subjects resolved symptoms in 11 days on average, and oxygen saturation improved in 24hrs (87.4% to 93.1%, p=0.001). Hospitalizations and deaths were significantly fewer (p<0.002 or 0.05, respectively) than in background-matched controls from the CDC database. Triple combination therapy is safe and effective even in moderate-severe patients with hypoxia treated in the outpatient setting.

Trial Registration: N/A, see methods.

Keywords: SARS-CoV-2, COVID, Coronavirus, Ivermectin, Doxycycline, Zinc

List of abbreviations:

χ^2 test (Chi-Square Test); CDC (Center for Disease Control); CI (Confidence Interval); COVID-19 (Coronavirus, SARS-COV-2); Hrs. (Hours); HAZDpaC(Hydroxychloroquine,Azithromycin,Vit D, Zinc Pack); *H. pylori* (*Helicobacter pylori*);ICT (Ivermectin Combination Therapy); PMH (Past Medical History) ; pt (point); pts (points); SEM (Standard Error of Mean); SOB (Shortness of breath); SpO_2 (Percent Saturation Peripheral Oxygen); temp (Body temperature in ^{0}F)

Declarations:

Ethics approval and consent to participate: All subjects were explained the study and provided written informed consent. This study was approved by E&I Review Services (https://www.eandireview.com/) as study # 210006.

Consent for publication: All authors have read and agreed to the final submitted contents of this publication.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding: This study was funded by ProgenaBiome, LLC.

Acknowledgements: The authors would like to thank Margaux Alvaran for technical assistance.

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Introduction

There is currently a lack of effective treatments for early or ambulatory patients with COVID-19. Patients testing positive are sent home to isolate and generally, no specific line of treatment is prescribed in this phase of the illness. However, there is growing evidence that certain repurposed drugs with good safety profiles, taken early, can significantly improve outcomes and even avoid or delay the need for immune-modulators, antiplatelet / antithrombotic therapy, and the administration of oxygen [1].

Among the most extensively studied of such COVID-19 therapeutics is ivermectin (IVM), a drug that has been used safely in 3.7 billion doses worldwide since 1987 [2-4]. Recently, Dr. Satoshi Omura, the 2015 Nobel prize co-laureate for the discovery of IVM, and colleagues comprehensively reviewed studies to date on IVM activity against COVID-19, concluding that the preponderance of the evidence demonstrated such efficacy [2]. IVM has been tested in more than 20 randomized controlled trials (RCTs) for COVID-19 treatment, with statistically highly significant clinical benefits in almost all of these and a pooled mortality reduction of 78% for the treatment vs. control groups [5]. Five such studies for IVM treatment of COVID-19 recently published in top-tier medical journals have all shown multiple clinical benefits for IVM vs. controls, most of these with high statistical significance on the order of $p < 0.002$ [6-10]. IVM is well tolerated at much greater than the standard single dose of 200 μg/kg [11,12] and has been used in RCTs for COVID-19 treatment at cumulative doses of 1,500 μg/kg [13], 1,600 μg/kg [14], and 3,000 μg/kg [15] over 4 or 5 days either without or with mild and transient adverse effects. Not surprisingly, IVM has become extensively used in the prevention and early disease management of COVID-19, particularly in non-Western countries.

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Despite this strong evidence of clinical benefit in COVID-19 for IVM therapy, variation in therapeutic regimens especially with respect to addition of a broad spectrum antibiotic and zinc, has led to confusion as to how best to manage acute infections. Indeed, the most impressive of the early ambulatory multi-drug therapy, claiming 87% and 75% reductions in hospitalization and deaths, respectively, both with a P value of 0.001, in 869 high risk subjects, left optimal management strategy unclear due to a mixed use of IVM and hydroxychloroquine (HCQ) [16]. There is an immediate need for an effective, safe, and practically available combination therapy formulated on the basis of the best available data.

At a cellular level, IVM modulates communication between the cytoplasm and nucleus, creating a hostile environment for assemblage of virus, while at the same time reducing cytokine-mediated inflammation. In addition, IVM inhibits pathology following infection with the COVID-19 virus, by specifically blocking binding of the virus “Spike” protein to the ACE2 receptor. Finally, IVM has been associated with favorable changes in cellular innate immunity [17].

Our group has been developing antiviral drug combinations for COVID-19 and found IVM to be particularly effective as a co-therapy for use early in COVID-19 to shorten the time to symptom resolution and to prevent hospitalization. IVM used alone can at times be only partially effective but not curative [6,1,19] yet a higher dose of IVM plus azithromycin and zinc has achieved a 92% mortality reduction vs. controls [14]. Thus, we chose a combination of safe and widely available medications, approved for other indications and without drug-drug interactions or QT prolongation that inhibits intracellular virus replication and possess some anti-inflammatory properties.

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The use of combination therapy for intracellular bacterial infections is not new and has been used successfully to treat Tuberculosis, *Helicobacter pylori* infections, leprosy and intracellular viral infections such as *Hepatitis B & C* - where a single component of the combination therapies is rarely curative. In some viral infections e.g. HIV, even combined multiple antiviral drugs cannot completely cure but suppress the viral load perpetually. IVM is best known for its broad-spectrum efficacy for parasite infections, its high cure rate and limited drug resistance when used in combination [20]. Although useful, IVM used alone is not the 'magic bullet'. Combinations can help lower individual doses and reduce side effects. To cover all age group requirements, we combined IVM with doxycycline and zinc as active components and with Vitamins D and C as replacement 'excipients' given to supplement common clinical deficiencies in the aged.

This study reports the use of the above combination therapy in consecutive, ambulatory, complex, at times profoundly hypoxic patients whose oxygen saturation (SpO_2) was as low as 73%. Participants were treated by an experienced clinical trials team within Ventura Clinical Trials Inc..

Methods:

Subjects:

Subjects were identified from patients referred by physicians, or word-of-mouth in Los Angeles, Ventura County, CA, and other states in the USA. These patients were referred to participate in clinical trials under clinicaltrials.gov ID NCT04482686 (which is a double-blind Randomized Control Trial). However some did not qualify for this trial as their oxygen saturation was less than 90%, and were deemed too sick to enter a placebo-controlled trial. Given they were excluded and refused to go to the hospital, they were treated off-label via telemedicine, during Aug. 2020 and Feb. 2021. Subjects were given the opportunity to participate in this open label

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trial with IRB oversight once the diagnosis was made via swab RT-qPCR testing once the diagnosis was made via swab RT-qPCR testing. Inclusion criteria were as follows 1) positive PCR for COVID-19; 2) informed consent; 3) age $\geq$ 18 years, and 4) agreement to practice two highly effective methods of birth control if of childbearing potential. Exclusion criteria included 1) allergies or drug interactions with the combination therapy components; 2) listed comorbidities, including seizure risk; and 3) pregnancy.

Treatment:

Treatment began as soon as practical, within 72 hrs. of patients presenting to Ventura Clinical Trials. All screened subjects met the inclusion criteria and were enrolled consecutively. Treatment was defined as 'IVM Combination Therapy' (ICT) and consisted of ten days of oral: Doxycycline (100mg twice a day), IVM (12mg on day 1, day 4, and day 8), Zinc (25mg twice a day), Vitamin D_3 (1500 IU twice a day) and Vitamin C (1500mg twice a day). ICT was given daily for ten days only.

Two patients (#10 and #23) received an initial treatment on day one of 36 mg IVM (rather than 12mg) due to particularly low SpO_2 or expected clinical need.

Monitoring:

Subjects self-recorded symptoms in their daily logs (supplementary 1) for the first 10 days. Electrocardiograms (EKGs), blood pressure, temperature (reported in ^0F) and SpO_2, were collected via provided medical equipment at home. On days 1, 5, 10 and 30, SARS-CoV-2 testing swabs were self-collected by subjects and sent to pathology for testing. Pregnancy tests were performed as appropriate.

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Endpoints:

Endpoints were 1) time from presentation to negative SARS-CoV-2 PCR; 2) time from presentation to symptom resolution; 3) progression to hospitalization; 4) patient survival.

Externally Controlled Trial (ECT) Arm:

Given the challenges for COVID-19 of enrolling high risk severely hypoxic patients in an open-label trial with an untreated control arm, our treated group arm survival was compared to the control group survival rate in the general population. This ECT, also known as a synthetic control arm, was calculated from the public CDC database of COVID-19 subjects [21]. Available information includes age range, presence of any chronic condition (COVID-19-vulnerability or otherwise, conditions not specified), date of infection, and whether the COVID-19 diagnosis was laboratory-confirmed. We used information from all subjects who met the following criteria: 1) age 50+ years; 2) laboratory-confirmed COVID-19 diagnosis; 3) death/survival, race, and sex status available and known; 4) infection prior to March 2021; and 5) subject had any co-morbidities. This synthetic control arm development was carried out after our clinical data were obtained, and so selection criteria chose the control subjects closely matched to the subjects in our study, all of whom had some underlying condition and a large majority were over 50 years of age. The CDC database was analysed using CSViewer vs. 1.3 (EasyMorph Inc, Toronto, ON, CA, http://easymorph.com).

Covidex calculations and statistics:

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Covidex and Covidex-F are ambulatory SARS-CoV-2 infection disease severity measures that we developed and validated in this study. They are weighted particularly to emphasize SpO_2, and Covidex-F includes a variable for body temperature.

Covidex score = 1 pt. (if history of sleep apnea) + 1 pt. (if history of COPD) + 1 pt. (if history of cardiovascular disease) + 1pt. (if history of asthma) + 1pt (if history of prior clots, ischemia or stroke) + 1pt (if obese, i.e. 30 kg/m^2 < BMI < 40 kg/m^2) + 2pts (if severely obese, i.e. BMI $\geq$ 40) + 1pt (if age $\geq$ 60 years) + [95-(SpO_2 as a percentage)]pts. For instance, a hypothetical patient with a history of asthma and morbid obesity with a SpO_2prior to treatment of 85% would have a Covidex score of 1 (for asthma) + 2 (for obesity) + 10 (for $SpO_2$of 85%) = 12 pts.

Covidex-F score = Covidex score + 1 pt. (if temperature on presentation between 99.5^0F and 100.4^0F) + 2 pts. (if temp on presentation between 100.4^0F and 103.5^0F) + 3 pts. (if temp on presentation $\geq$ 103.5^0F).

Best-fit lines were made to assess the correlation between Covidex scores and time from treatment to symptom resolution. Regression was carried out in Prism version 8 (GraphPad Prism software for Windows, San Diego, California USA, www.graphpad.com) using least square regression without weighting or special handling of outliers. All graphs were prepared by and statistical analysis done using GraphPad Prism version 8, and the error bar are indicative of the standard error of the mean (SEM).

Results:

Table 1 lists all subjects in the study, two of which did not consent to ICT treatment (subjects #10 and #26), and their associated race, gender, symptoms, fever, and other clinical notes. All subjects had COVID-19-related symptoms on presentation, and the symptom range was broad with several showing shortness of breath (SOB). The vast majority of subjects, 21 of 24

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(87.50%), had fever on presentation with a mean temperature for all 24 subjects of 101.2 $\pm$ 0.32 ^{0}F. Specifically, 1/24 (4.17%) had low grade fever (99.5-100.4), 18/24 (75.00 %) had medium grade fever (100.5-103.4), and 2/24 (8.33%) had high grade fever ($\geq$ 103.4) ^{0}F.

Table 2 summarizes the demographics and past medical history (PMH) of subjects who consented to treatment (total n=24, 2 additional subjects who declined treatment are excluded). Notably, patients were older (a known COVID-19 vulnerability) with a mean age of 66 $\pm$ 2.75 years, and a range of 43 to 94 years (Table 2A). The population of the 24 subjects consenting to treatment (not subjects #10 and #26, Table 1) was 63% males. Death of untreated subjects #10 and #26 excluded downstream analysis in the other figures.

Table 2B lists the number of patients who had comorbidities associated with COVID-19 vulnerability, based on CDC guidelines[22]. These comorbidities are chronic kidney disease, chronic obstructive pulmonary disease (COPD), Down syndrome, cardiovascular disease, immunocompromised state including HIV, obesity (body mass index [BMI] of 30 kg/m^2 or higher but $<$ 40 kg/m^2), severe obesity (BMI $\geq$ 40 kg/m^2), pregnancy, sickle cell disease, smoking, type 2 diabetes. Of note, no subjects had cancer, Down syndrome, or sickle cell disease and none were pregnant nor were smokers.

Many subjects had multiple comorbidities associated with COVID-19 vulnerability, as outlined in Table 2B. In total, 11/24 (45.83%) subjects had COVID-19-vulnerable comorbidities of which 3 (12.50%) had 2 separate comorbidities, and 2 (8.33%) had 3 co-morbidities.

A minority of subjects (n = 7) had other COVID-19 treatment(s) prior to and/or during ICT administration, namely Remdesivir (n = 1 subject), involvement in a placebo-controlled trials of HAZDpaC (n = 4 subjects, trial clinicaltrials.gov NCT04334512; may have been given treatment or placebo) and hydroxychloroquine (HCQ, n = 3 subjects).

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Fig. 1 demonstrates all subjects recovered from COVID-19, typically within one to two weeks. Fig. 1A shows various durations for each subject and average values (one outlier excluded). Time from onset of symptoms to treatment initiation is shown in column 1 and averages 9.2 $\pm$ 2.1 days. Time from start of treatment to symptom resolution was11.6 $\pm$ 1.4 days. Time from first positive to first negative PCR was 16.9 $\pm$ 1.6 days and is less than three weeks. Time from start of treatment to first negative PCR was 11.5 $\pm$ 1.6 days and is also less than three weeks.

Fig. 1B shows that 100% of subjects survived COVID-19, without need for hospitalization or ventilator use. As noted in Table 2, many of these subjects were older and with comorbidities. When compared to the synthetic control arm, derived from the CDC database (see methods, and as follows), this was a significant increase in survival rate (p = 0.044) and decrease in hospitalization rate (p = 0.0011), evaluated via χ^2 test. Of note, the patients in this CDC database likely did receive treatment, of an unknown nature. Thus, the survival rate of this synthetic control reflects the "typical" survival rate in the USA, which is significantly less than the 100% survival rate observed on ICT.

The 100% survival rate on ICT was compared with survival rates from the CDC database of COVID-19 subjects. 356,424 control subjects were obtained, based on qualification criteria described in the methods section. These criteria focused on older subjects (50+ years), similar to our study population, that also had underlying conditions. One should note that the underlying condition criteria information available in this database refers to chronic conditions of any type, whether or not the condition induces COVID-19-vulnerability. With this definition, 100% of control subjects and ICT-treated subjects had underlying conditions of any type.

Our critical finding in Fig. 2 (see also Table 2), showed that 23/24 patients were hypoxic with SpO_2 < 90%. Some subjects consenting to treatment had SpO_2 as low as 73%, 77%, 84% and

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85% on presentation. As a whole, shown in Fig. 2A, the SpO_2 of subjects was significantly less than 95%, the defined point of cure (95% CI of mean SpO_2= 85.5 to 89.4, Mean = 87.4 $\pm$ 0.93%). Subjects' SpO_2 increased within 24 hrs. of treatment. Their mean SpO_2 before treatment (for subjects with data before and after 24 hrs.) was 86.5% $\pm$ 1.3, and after 24 hrs. of treatment, 93.1% $\pm$ 0.63, a highly significant and rapid increase ($p < 0.001$). SpO_2 then continued to rise. Treatment continued for 10 days reaching the point of successful treatment or cure, which was $SpO_2 > 95\%$. Successful treatment was reached for all subjects. That is, there was a 100% restoration rate in terms of SpO_2. No patient who accepted treatment required hospitalization.

Two subjects (#10 and #26) declined treatment. These subjects did not recover SpO_2 and died from COVID-19 infection. An adverse drug event of dizziness was reported by one subject, who nevertheless continued with treatment successfully otherwise.

Fig. 3 shows the validation of the Covidex and Covidex-F scores we developed, defined in our methods. These scores provide an index of COVID-19 predicted severity, based on past medical history, O_2 just before treatment and fever grade on presentation. The mean Covidex score was 10.34 $\pm$ 1.08 and the mean Covidex-F score was 11.63 $\pm$ 1.13, with 87% and 85% of the score points, respectively, coming from the contribution of the SpO_2 term. In other words, approximately 80 to 90 % of this score is weighted towards SpO_2. It should also be noted, the PMH aspects that contribute to this score emphasize respiratory, cardiovascular, and obesity histories and differ from CDC defined COVID-19-vulnerabilities.

Covidex and Covidex-F are both plotted vs. time from treatment to symptom resolution. Both shows statistically significant correlations (Covidex: $p = 0.0096$, $r^2 = 0.2078$; Covidex-F: $p = 0.0288$, $r^2 = 0.2790$), indicating that either Covidex or Covidex-F is associated with and may predict time to symptom resolution.

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Discussion:

We report for the first time a highly effective ICT which led to 100% survival and cure in unselected ambulatory 'moderate to severely' ill COVID-19 patients with hypoxia managed as outpatients. Given our experience developing combination therapies for *H. pylori*, we trialed a number of different IVM-based combinations on ambulatory COVID-19 patients, searching for a cure, and found the above combination to be the best as a 'foundation therapy' for COVID 19. Understanding that a personalized approach may require added HCQ or other components in some patients much like *H.pylori* resistant to triple therapy sometimes requires quadruple therapy. Hypoxia is a demonstrated predictor of COVID-19 mortality [23]. For example, several of these patients had profound hypoxia, measured by oximetry, at 73%, 77%, 84% and 85% on presentation. Despite a symptom to treatment delay of 9.2 days, our treatment brought rapid improvement – beginning in some within 12 hrs. with a mean SpO_2 rising from 86.5 to 93.1 in the first 24 hrs. There was a parallel improvement in the symptoms including loss of cough, fever and tiredness. Also, the time from the start of treatment to the first negative PCR averaged 11.5 $\pm$ 1.6 days. Generally, such ill patients would have been admitted to the hospital, yet all those treated with the ICT avoided hospitalization and none died.

Turning to the ECT 'synthetic control arm' it is clear that the ICT was statistically superior to the control arm even though a small patient group was reported. The very low adverse effects from reported studies and this treatment group supports the use of ICT if clinical symptoms and risk factors for COVID-19 progression are present, even in cases with PCR pending results. ECT

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arms are now increasingly used, especially where the control arm or 'standard-of-care' arm may have a fatal outcome[24].

The rationale for using combination antiviral therapy is based on our growing understanding that intracellular infections – bacterial or viral, cannot be reliably cured using a single drug. It is also based on our knowledge that IVM resistance is common. Hence, there is no single 'silver bullet' for COVID-19, and the indiscriminate use of IVM *alone* could induce COVID-19 resistance by generating drug-resistant strains. Resistance to IVM was the case when used alone in scabies, nematodes, strongyloidiasis, microfilaridermias, onchocerca and volvulus [19,25-31]. We are now seeing resistance develop even in combined therapies using two antibiotics for *H. pylori*. This teaches us to use IVM in COVID-19 only in combination therapies, especially so, with growing reports of mutant strains resulting in vaccine breakthrough infections [32]. Hence, the proposed combination therapy was developed to induce *cure* more rapidly, *prevent* resistance, and *overcome mutant strain* emergence - no replication no mutation.

Regarding strategies in the development of combination therapies, intracellular coronavirus replication requires several active drugs to inhibit viral replication. IVM, doxycycline and zinc all individually inhibit coronavirus replication and, although there are other candidates, we have proposed the above combination based on efficacy, component safety profiles, inexpensive nature, and lack of drug-drug interaction. The combination of IVM and doxycycline has also been demonstrated to act in synergy against COVID-19 [33]. This combination also appears to overcome the need for high doses of IVM identified by Caly and colleagues when used alone[34]. Further, given that zinc plays a key role in antiviral activity [34536] it would combine well with the ionophores (IVM and doxycycline) to increase its intracellular concentration and expedite viral clearance [37]. We have also assessed drug-drug interactions and found that the

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combination of zinc with IVM and doxycycline has no reported interactions. Additionally, each of these drugs has a low adverse side effects profile and no QT prolongation as reported with azithromycin.

Overall, based on the current literature, a 10-day combination therapy of IVM, doxycycline and zinc will not only improve symptoms [6,7] but also accelerate recovery from COVID-19. We have chosen a safe IVM dosage approved for parasites of 36mg over 10 days, and this dose has been shown to be both effective and safe in COVID-19 treatments [38]. The staggered IVM dosage over 10 days is proposed based on the half-life clearance of the drug in plasma (up to 66 hrs.)[39]. The proposed duration would allow constant availability of adequate plasma level IVM to facilitate zinc entry into the cells. Hence, the above rationale explains why some publications have already shown that IVM alone is not adequate to cure COVID-19 [6,18,19] while a multidrug regimen is likely to be more efficacious [40].

While underpowered, this study enrolled *consecutive subjects* into study and did not bias subject selection from different time points. Many of the enrolled subjects were profoundly ill with subjective assessments that may have resulted in hospital admission and/or intubation. Yet, ICT anti-viral activity appears to have rapidly restored SpO_2 and reversed other symptoms which could not be explained simply by the developing immunity.

A weakness of this study is the lack of a concomitantly enrolled control arm. However, given the potential fatal outcomes Tess Lawrie et al. indicated [41] *'it is no longer ethical to approve the use of a control arm as so many profoundly ill patients in the control arm would die'*, as did our two subjects who declined treatment [42]. Hence our study has made use of the ECT or 'synthetic' control arm which has enabled us to make matched age and comorbidity comparisons. Institutional Review Boards should now include the provision to include available

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synthetic arms and reject trials that include in a COVID-19 trial a control arm as published by Lawrie[41].

This study builds on an extensive literature, to provide a practical inexpensive, safe, readily available and highly effective triple therapy aiming to prevent resistance and one that can confidently be used as a routine treatment for outpatient COVID-19.

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Figure and table legends:

Table 1: Listing of subjects and COVID-associated symptoms on presentation and other characteristics. O_2% Sat = SpO_2 just before time of treatment initiation. O_2% Sat post 24 hrs. = SpO_2 24 hrs. after treatment initiation. Rx Start date = Day 1 of ICT administration. SOB = Shortness of Breath

Table 2. A. Age and demographics of subjects. B. Prevalence of COVID susceptible co-morbidities. C. Number of concurrent comorbidities in subjects.

Figure 1: Complete recovery was seen in all patients within 1 to 3 weeks.
A. Time in days to various stages of symptom onset and resolution.
Nearly all subjects resolved symptoms and became PCR negative in 3 weeks
Col 1: Symptom onset to start of treatment (n = 23, mean = 9.17 $\pm$ 2.05)
Col 2: Start of treatment to resolution of symptoms. (n = 23, mean = 11.61 $\pm$ 1.38)
Col 3: First PCR positive to first PCR negative (n = 10, mean = 16.90 $\pm$ 1.58)
Col 4: Start of treatment to first PCR negative (n =10, mean = 11.50 $\pm$ 1.60)
B. Top, 100% survival rate was seen in patients, which is significantly higher ($p = 0.044$ via χ^2 test) than synthetic control from CDC database of equivalent or less COVID-vulnerable subjects. Bottom, No (0%) patients required hospitalization, which is significantly less ($p = 0.0011$ via χ^2 test) than synthetic control from database.

Figure 2. A. O_2 saturation prior to treatment was significantly ($p < 0.05$) less than 95%, the defined successful treatment reached by all subjects. B. O_2 saturation significantly increased in subjects 24 hours after treatment (paired *t*-test, $p < 0.001$, only subjects with data before and after treatment included). O_2 saturation continued to rise, and until the defined cure of greater than 95% O_2 saturation.

Figure 3: Ambulatory COVID scores, Covidex and Covidex-F (see methods for definition; Covidex-F includes fever measure) vs. time from start of treatment to symptom resolution. There

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was significant relation between either Covidex score (Covidex, $p = 0.0288$; Covidex-F, $p = 0.0096$) and treatment resolution time.

Supplementary Figure 1: Example of symptom log sheet used by subjects.

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Figures and Tables:

Table 1:

ID	Age	Race	Gender	Symptoms	Temp. (˚F)	O2 % sat.	O2 % sat. post 24 hrs.	Rx. start date	Symptom resolution date	PCR pos. date	PCR neg. date
1	65-69	caucasian	M	runny nose, sore throat, dizzy, low energy	99.3	90	94	12/nn/2020	12/nn/2020	11/nn/2020	1/nn/2021
2	60-64	caucasian	M	SOB, chest congestion, productive cough, nausea, vomit	105	77	87	12/nn/2020	12/nn/2020	11/nn/2020	12/nn/2020
3	75-79	caucasian	M	low energy	101	88	96	10/nn/2020	11/nn/2020	10/nn/2020	10/nn/2020
4	65-69	caucasian	F	loss of appetite, cough, chills, SOB	101	97	96	10/nn/2020	10/nn/2020	10/nn/2020	10/nn/2020
5	65-69	caucasian	F	vomit, weak, body aches, anosmia	101	89	95	12/nn/2020	12/nn/2020	12/nn/2020	n/a
6	40-45	caucasian	F	PE, headache, body ache, cough	101	88	94	1/nn/2021	2/nn/2021	1/nn/2021	2/nn/2021
7	60-64	caucasian	M	productive cough, headache	102	86.5	91	11/nn/2020	12/nn/2020	11/nn/2020	12/nn/2020
8	55-59	caucasian	M	cough, nasal congestion, SOB, body ache	102	88	96	10/nn/2020	11/nn/2020	10/nn/2020	11/nn/2020
9	90-94	hispanic	F	low energy, SOB, confusion, loss of appetite, shaking	102	88	94	1/nn/2021	1/nn/2021	12/nn/2020	n/a
10	65-69	hispanic	M	cough, SOB, respiratory failure	100.6	72	87	declined	death	12/nn/2020	n/a
11	60-64	hispanic	F	cough, SOB	102	90	96	1/nn/2021	1/nn/2021	12/nn/2020	n/a
12	45-49	hispanic	M	SOB	104	84	91	12/nn/2020	12/nn/2020	12/nn/2020	n/a
13	65-69	caucasian	F	cough, congestion, rash	102	88	91	11/nn/2020	12/nn/2020	11/nn/2020	n/a
14	65-69	caucasian	M	post-nasal drip, cough, sinus pain	98	88	91	11/nn/2020	12/nn/2020	11/nn/2020	n/a
15	70-74	hispanic	M	low energy, productive cough, anosmia	101	88	n/a	12/nn/2020	1/nn/2021	12/nn/2020	n/a
16	65-69	hispanic	F	dry cough, body aches, low energy, anosmia	100	88	n/a	12/nn/2020	1/nn/2021	12/nn/2020	n/a
17	45-49	caucasian	F	diarrhea, rash, renal pain	102	87	94	8/nn/2020	8/nn/2020	7/nn/2020	n/a
18	85-89	caucasian	M	cough, fever, low energy	102	88	95	1/nn/2021	1/nn/2021	1/nn/2021	1/nn/2021
19	55-59	caucasian	F	stomach pain, diarrhea, cough, rash	102	90	95	9/nn/2020	9/nn/2020	8/nn/2020	n/a
20	50-54	muslim	M	cough, fever, loss of appetite, chills	101.2	88	n/a	10/nn/2020	10/nn/2020	10/nn/2020	10/nn/2020
21	90-94	caucasian	M	low energy	102	85	91	2/nn/2021	2/nn/2021	2/nn/2021	2/nn/2021
22	60-64	hispanic	M	cough, low energy, loss of appetite	101.3	90	96	2/nn/2021	2/nn/2021	2/nn/2021	n/a
23	55-59	hispanic	M	cough, SOB	98	73	90	1/nn/2021	2/nn/2021	2/nn/2021	1/nn/2021
24	45-49	hispanic	F	chest pain, SOB	98.6	90	n/a	2/nn/2021	2/nn/2021	2/nn/2021	n/a
25	85-89	hispanic	M	severe SOB, low energy, trouble walking	101.6	90	95	2/nn/2021	3/nn/2021	2/nn/2021	n/a
26	85-89	caucasian	M	SOB	102	88	n/a	declined	death	10/nn/2020	n/a

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Table 2:

A.

Age (mean, SEM, range)	66, 2.75, 43 - 94
Male (n, %)	15, 62.5%
Female (n, %)	9, 37.5%
Race (n, %)	
Caucasian	14, 58%
Hispanic Mexican	7, 27%
South American	2, 8%
Other	1, 4%

B.

Comorbidity	**# of subjects**	**% of subjects**
Type 2 diabetes mellitus	6	25.00
Heart or cardiovascular	5	20.83
COPD	3	12.50
Obesity (BMI 30 - 40)	3	12.50
Severe Obesity (BMI $\geq$ 40)	2	8.33
Chronic kidney disease	1	4.17
Immunocompromised state	1	4.17

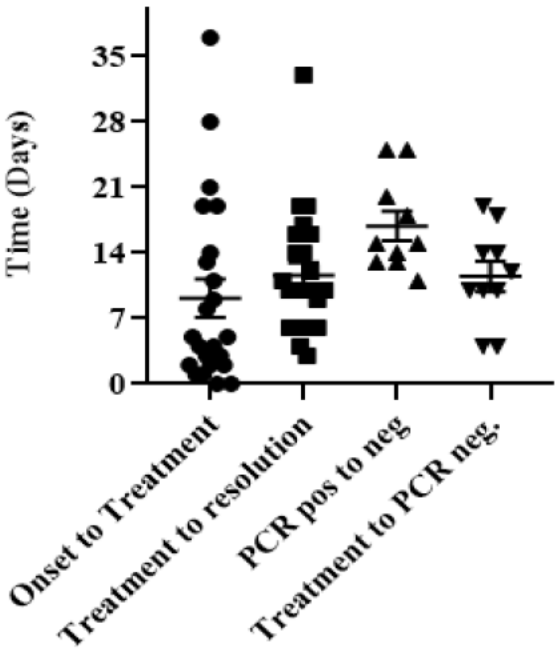
C.

# of co-morbidities	**# of subjects**	**% of subjects**
0	13	54.17
1	6	25.00
2	3	12.50
3	2	8.33

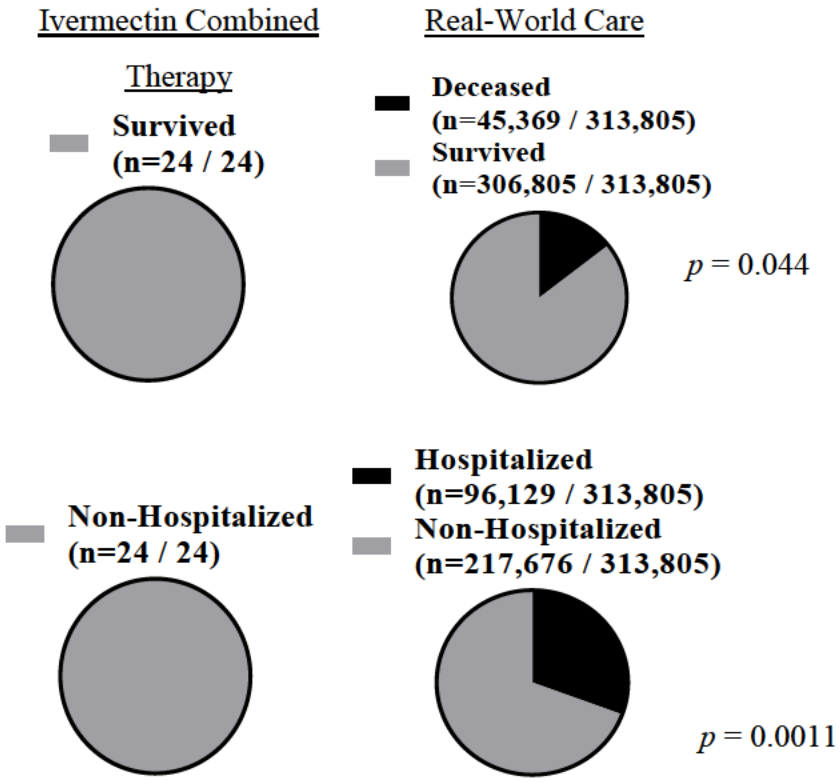
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Figure 1:

A.



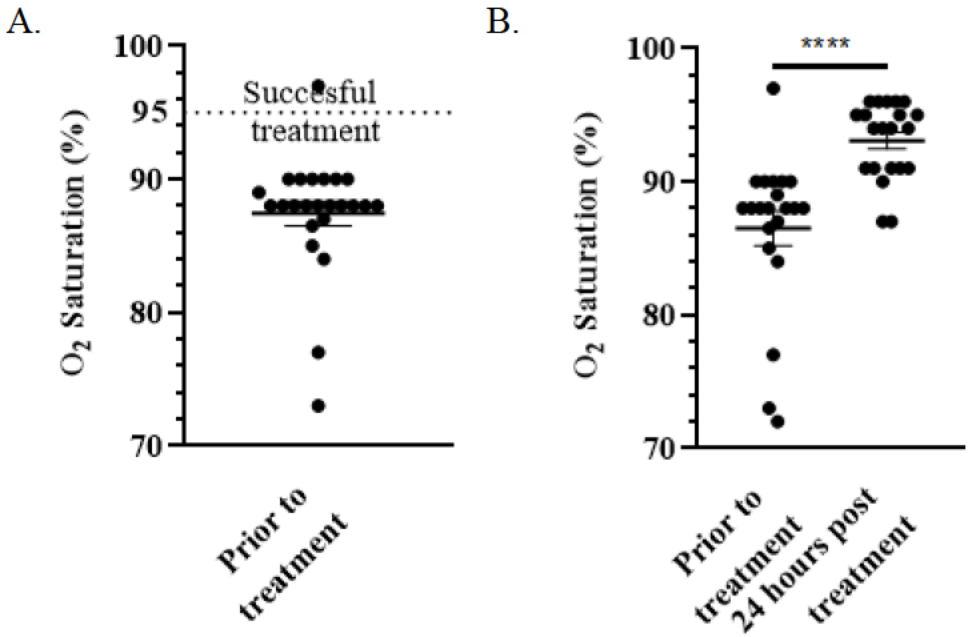
B.



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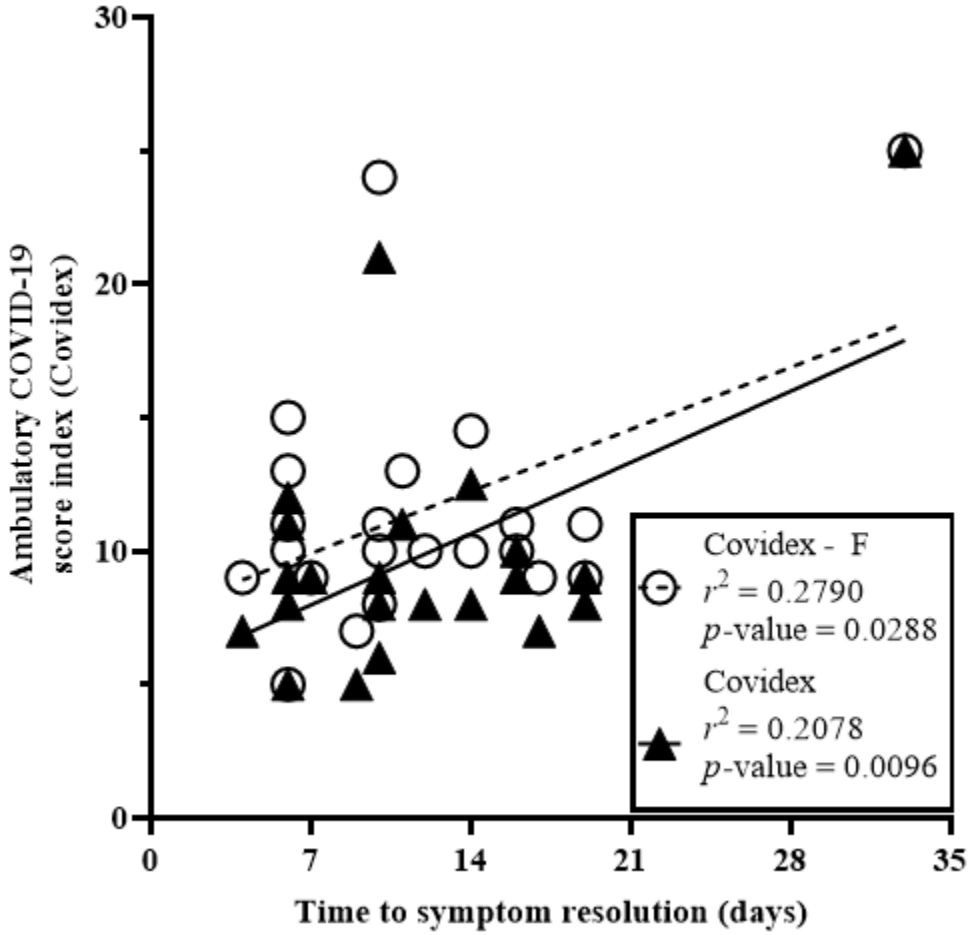
Figure 2:



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Figure 3:



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Supplementary Figure:

Supplementary Figure 1:

Sponsor: ProgenaBiome
Protocol: PRG-049
Principal Investigator: Sabine Hazan, M.D.

OPELLA THERAPEUTICS

Patient Number: PRG - 049 - ___ ___ ___
Patient Initials: ___ ___ ___

Symptom Tracker

Indicate if patient symptoms (+) Improved, (-) Worsened, or (/) Not Change. Indicate "N/A" if common symptoms included in chart are not present

Initial Test Date: _____

	SYMPTOM	START DATE	Day 1	DAY 3	DAY 5	DAY 7	DAY 10	DAY 30	RESOLUTION DATE
1.	Fever								
2.	Cough								
3.	Loss of appetite								
4.	Loss of smell								
5.	Loss of taste								
6.	Diarrhea								
7.	Fatigue								
8.	Shortness of Breath								
9.	Headache								
10.									
11.									
12.									
13.									
14.									
15.									

Page ___ of ___

Version Date: 29APR2020 Version 3.0

Safety and efficacy of a MEURI Program for the use of high dose ivermectin in COVID-19 patients

Short Title: A MEURI Program with ivermectin for COVID-19

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Keywords: ivermectin; COVID-19; mortality; ICU admission; safety

Abstract

Background

In the absence of antiviral alternatives, interventions under research for COVID-19 might be offered following guidelines from WHO for monitored emergency use of unregistered and experimental interventions (MEURI). Ivermectin is among several drugs explored for its role against SARS-CoV-2, with a well-known safety profile but conflicting data regarding clinical utility for COVID-19. The aim of this report is to inform on the results of a MEURI Program of high-dose ivermectin in COVID-19 carried out by the Ministry of Health of the Province of La Pampa, Argentina.

Methods

COVID-19 subjects, within 5 days of symptoms onset were invited to participate in the program, which consisted in the administration of ivermectin 0.6 mg/kg/day for 5 days plus standard of care. Active pharmacosurveillance was performed for 21 days, and hepatic laboratory assessments were performed in a subset of patients. Frequency of Intensive Care Unit (ICU) admission and COVID-19-related mortality of subjects in the ivermectin intention to treat group were compared with that observed in inhabitants of the same province during the same period not participating in the program.

Results

From 21232 subjects with COVID-19, 3266 were offered and agreed to participate in the ivermectin program and 17966 did not and were considered as controls. A total of 567 participants reported 819 adverse events (AEs); 3.13% discontinued ivermectin due to adverse. ICU admission was significantly lower in the ivermectin group

compared to controls among participants ≥40 year-old (1.2% vs 2.0, odds ratio 0.608; p=0.024). Similarly, mortality was lower in the ivermectin group in the full group analysis (1.5% vs 2.1%, odds ratio 0.720; p=0.029), as well as in subjects ≥ 40 year-old (2.7% vs 4.1%, odds ratio 0,655; p=0.005).

Conclusion

This report highlights the safety and possible efficacy of high dose ivermectin as a potentially useful intervention deserving public health-based consideration for COVID-19 patients.

Introduction

COVID-19 constitutes a public health emergency at a global scale since its appearance in Wuhan, China, in December 2019 (1). By August 2021, over 200 million cases and 4 million deaths have been reported worldwide (2). Vaccine rollout campaigns, which currently offer the best hopes for pandemic control, are a key targeted pharmacologic intervention for containment of disease spread and impact on the incidence of severe cases (3,4).

Despite having an asymptomatic or mild course in most cases, COVID-19 constitutes a significant burden on health systems unprepared to cope with outbreaks requiring, among other things, massive testing capacity for rapid case detection and isolation, expansion of intensive care unit (ICU) capacity and case management guidelines for a previously unknown pathogen. This public health crisis has been, and still is, more profound in countries with weaker health systems (5).

The unprecedented progress in vaccine development has not been matched by the development of antiviral molecules, either new or repurposed, that could contribute to the treatment or prevention of COVID-19. With convalescent plasma, monoclonal antibodies, hydroxychloroquine and antiretrovirals among many molecules tested in-vitro and in observational and clinical trials, different treatment guidelines only agree in the use of corticosteroids, thromboprofilaxis and respiratory support, none of them an antiviral, in their recommendations (6,7).

Ivermectin (IVM) is an endectocide drug widely used for the treatment and control of onchocerciasis and lymphatic filariasis through mass drug administration programs, which has a wide therapeutic index and a benign safety profile (8,9). Besides its known

uses, it has been evaluated as an antiviral, demonstrating in vitro activity against zika, rabies and dengue among other viruses (10). In the case of dengue, a recently published randomized clinical trial from Thailand showed positive although inconclusive results (11). For SARS-CoV-2, early on the pandemic, the report of the antiviral activity of IVM in Vero cells cultures sparked widespread interest in the potential utility of this oral, safe and affordable drug against COVID-19 (12). However, after over a year of several publications addressing this question, there is a lack of clear evidence for or against the use of IVM in COVID-19 patients (6,13). With at least two completed double-blind randomized clinical trials (RCTs) showing no effect in clinical endpoints, other smaller randomized trials using higher doses identified significant antiviral effects (14–18). That undefined landscape is summarized by the current NIH COVID-19 treatment Guidelines stating that there is insufficient data to recommend either for or against the use of IVM in COVID-19 patients (6).

In 2016, the World Health Organization (WHO) issued the Guidance for Managing Ethical Issues in Infectious Diseases Outbreaks, with the aim of complementing existing guidance on ethics in public health in situations of great uncertainty and including recommendations for the use of unproven interventions outside clinical trials, which based on a WHO response developed in the context of the outbreak of Ebola Virus Disease in Western Africa in 2014 are called “monitored emergency use of unregistered and experimental interventions” (MEURI) (19). These interventions apply when no proven effective treatments exist, it is not possible to initiate clinical trials immediately, existing preliminary data supports the intervention, relevant regulatory, ethical and scientific authorities approve such use, resources are available to minimize risks and patient´s informed consent is obtained. Proper monitoring and timely sharing

of the results with the wider medical and scientific community are also requirements to MEURI activities.

The aim of this report is to inform about the satisfactory safety and efficacy results of a MEURI Program for the use of high dose IVM in COVID-19 patients, carried out by the Ministry of Health of the Province of La Pampa, in the Patagonian region of Argentina.

Methods

MEURI Program for the use of high dose IVM in COVID-19 patients.

By the end of January 2021, the Ministry of Health of the Province of La Pampa (Argentina) authorized the implementation of a MEURI program based on the use of high dose IVM in COVID-19 adult patients (older than 18 year-old). In order to be able to participate, subjects had to be able to provide written informed consent, have a confirmed diagnosis of COVID-19 infection (by means of RT-PCR or antigen test) and symptoms onset within 5 days before entering the program. Exclusion criteria included pregnancy, breast feeding, hypersensitivity to IVM or acute allergic states, and active use of warfarin. Women with child-bearing potential were eligible if they were taking effective contraceptive measures before entering the program and agreed to continue with these measures for at least 30 days after receiving the last dose of IVM. Meanwhile ambulatory and inpatient subjects were allowed to participate (as long as they accomplished all inclusion criteria and had no exclusion criteria), admission to

ICU was considered an exclusion criteria. Every member of the health staff of the Province of La Pampa was instructed to invite to participate in the program to every COVID-19 patient identified within the first five days of symptoms onset. However, participation in the program was voluntary, and physicians could decide not to include subjects in the program based on their medical criteria.

Ethical approval was obtained from the Provincial Ethics Committee of La Pampa, and participating individuals provided written informed consent.

Intervention

Participants were evaluated at program entry with complete medical history and a brief physical exam. At the beginning of the program, safety laboratory assessments before and at the end of treatment were mandatory. However, after a preliminary safety analysis that triggered an amendment approved by the Ethics Committee, these assessments were no longer mandatory and could be performed or not, based on medical judgment.

Patients received oral treatment with IVM for 5 consecutive days within 30 minutes of food ingestion, preferentially of high fat content, at approximately 24 h intervals. IVM 6 mg, 9 mg and 18 mg tablets were used, combined to in all cases at a dose of 0.6 mg/kg/day based on baseline weight rounding to the lower full (6 mg) dose. There were no specific guidelines regarding medical management of COVID-19 infection for the participants in the IVM program, which was the same as for the rest of the population.

Safety Assessment

Active pharmacosurveillance was performed during the first 21 days after treatment start by means of the completion of a follow up chart, and safety assessment was based in all subjects that participated in the program in which follow up safety data was reported.

Hepatic safety assessment

Hepatic safety assessment was based on the analysis of hepatic lab exams performed before and after IVM treatment in a subset of patients, and consisted of laboratory determinations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin levels. Drug induced liver injury was defined according to the Latin American Association for Study of the liver definition, that includes (i) ALT elevation ≥5 ULN, (ii) ALP elevation ≥2 ULN (in the absence of known bone pathology driving the increase in ALP level) or (iii) ALT ≥3 ULN and simultaneous elevation of total bilirubin concentration above 2 ULN (20).

Efficacy analysis

In order to estimate the efficacy of the implementation of the program, the clinical evolution of the subjects in the IVM intention to treat (ITT) group was compared with that observed in inhabitants of the same province during the same analyzed period (from January 20, 2021 to May 20 2021) who did not participate in the program (control group, C). To identify them, the analysis of the National Health Surveillance System (SNVS 2.0) was used, which records, among other events, the notification of COVID-19 cases, their clinical and demographic characteristics and the respective laboratory studies, in a mandatory, nominal and immediate way, according to a national regulation.

Given that the registration methodology differs between the one used in the IVM-monitored intervention program and the one used for registering subjects and events in SNVS 2.0 database, it was decided to consider variables not dependent on the registration method in the system for the efficacy analysis. Specifically, the primary objectives of the evaluation were the analysis of the impact of the program on the frequency of ICU admission and COVID-19-related death. It should be noted that both ICU admission and death registration is carried out centrally, so their identification is independent of the type of follow-up carried out.

In order to compare the clinical course of both groups, subjects under 18 years of age and pregnant women were excluded from the analysis.

In the univariate efficacy analysis, subgroup analysis was performed according to age group (subjects ≥ 18 year old -whole sample- or subjects ≥ 40 year old), immunization status (excluding subjects with at least one vaccine dose) and mean IVM prescribed dose.

Statistical analysis-

Baseline characteristics of the two groups (C and IVM) were compared by means of Student`s T- test and Chi square. The clinical evolution was evaluated by Chi square Test and logistic regression analysis. Whenever possible, number needed to treat (NNT) values for IVM were estimated for the end points of ICU admission and death. The NNT values were estimated as the inverse of the difference in estimated absolute risk between control and IVM groups. In all cases, p-values <0.05 were considered statistically significant. All analysis were performed with GraphPad Prism version 9.1.0 for Windows (La Jolla, California).).

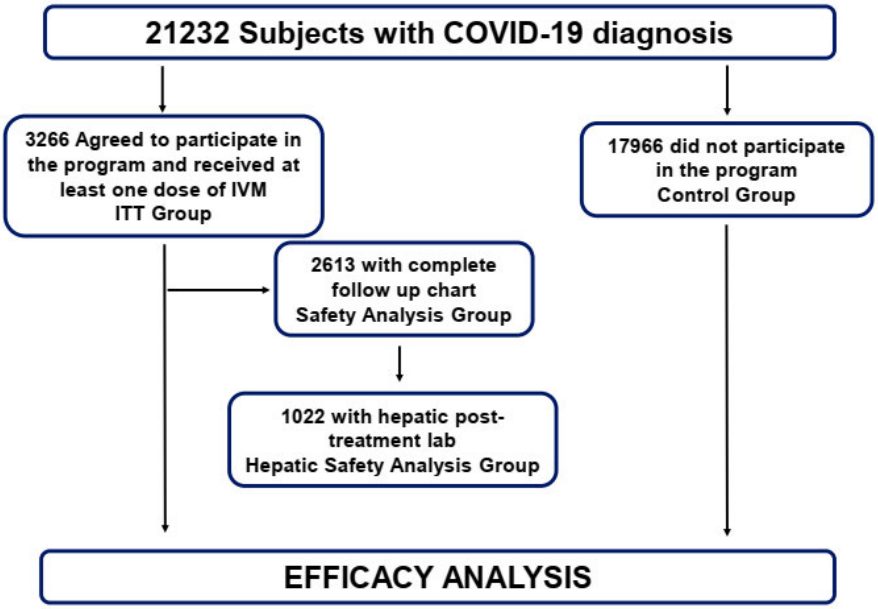
Results

Recruitment

A total of 21232 non-pregnant adults were identified as COVID-19 positive between Jan 20 2021 and May 20 2021. Of these, 3266 agreed to participate in the program and received at least one dose of IVM, and were included in the ITT analysis group. A group of 17966 subjects that did not participate in the program were included in the C group. Descriptive characteristics of the population are presented in Table 1.

Safety follow up data was obtained from 2613 subjects that participated in the program and were included in the Safety Analysis Group. Of these, 1022 were followed with post-treatment hepatic lab exams, and were included in the Hepatic Safety Analysis group (Fig 1).

Fig 1. Flow diagram of the MEURI Program for the use of high dose IVM in ambulatory COVID-19 patients.



224 **Table 1. Descriptive characteristics of the sample.**

VARIABLE	CONTROL GROUP (n=17966)	IVERMECTIN GROUP (n=3266)
AGE (years +/- SD)	42.30±16,65	43.81±15,47**
SEX	52.8% FEMALE 47.2% MALE	50.6% FEMALE# 49.4% MALE
COMPLETE IMMUNIZATION	323 (1.8%)	59 (1.8%)
INCOMPLETE IMMUNIZATION	1417 (8.0%)	271 (8.5%)
CARDIOVASCULAR DISEASE	1792 (10.0%)	344 (10.5%)
COPD	1177 (6.6%)	244 (7.5%)
HYPERTENSION	1583 (8.8%)	551 (16.9%)**

DIABETES	**1579 (8.8%)**	**315 (9.6%)**
NEOPLASM	**269 (1.5%)**	**63 (1.9%)**
OBESITY	**2241 (12.5%)**	**1182 (36.2%)****

COPD: Chronic obstructive pulmonary disease

Complete immunization: subjects with complete vaccine scheme at least 14 days before symptoms onset

Incomplete immunization: subjects with the first vaccine dose (of a two-dose scheme) received at least 14 days before symptoms onset

# p< 0,05 vs Control group

* p< 0,01 vs Control group

** p< 0,001 vs Control group

Safety Analysis

A total of 2613 participants were included in the safety assessment. Five hundred sixty seven (567) participants reported 819 adverse events (AEs). Eighty-two subjects (3.13

239 %) discontinued IVM due to adverse events and all AEs resolved after treatment
240 discontinuation. The most common AEs were diarrhea, followed by visual disorders,
241 dizziness, abdominal pain, headache and nausea (Table 2). Although many of the
242 symptoms presented during treatment resemble COVID, they were all assumed to be
243 related to IVM treatment.

244

245 **Table 2.** Safety analysis among subjects receiving ivermectin (n 2613). The table
246 shows adverse events reported in more than 0.5% of subjects

.ADVERSE EVENT	N (%)
Diarrhea	**155 (5.93%)**
Visual disorders	**136 (5.2%)**
Dizziness	**120 (4.59%)**
Abdominal pain	**91 (3.48%)**
Headache	**73 (2.79%)**
Nausea	**78 (2.98%)**

Anorexia	**31 (1.19%)**
Vomiting	**30 (1.15%)**
Heart rate elevation	**18 (0.69%)**
Rash	**16 (0.61%)**
Blood pressure elevation	**15 (0.57%)**
Pruritus	**14 (0.53%)**
Insomnia	**14 (0.53%)**
Drowsiness	**14 (0.53%)**

Hepatic safety analysis

Although there was a small but statistically significant increase in ALT, AST and total bilirubin values after IVM treatment (Table 3), among 1022 subjects that were followed up with laboratory determinations after IVM treatment, only one presented liver enzyme values compatible with low grade drug induced liver injury, that lead to drug discontinuation on day 4 of treatment. According to medical records, this subject had

abnormal baseline ALT and AST values (AST 240 U/l, ALT 375 U/l, Total Bilirrubin 0,63 mg/dl and the values peaked to AST 366 U/l ALT 630 U/l total bilirrubin 0.8 mg/dl and returned to AST 111 U/l, ALT 214 U/l and total bilirrubin 0.62 mg/dl. Considering the total hepatic safety sample (n=1022), this represents an incidence of 0.98/1000 treated subjects.

Table 3. Hepatic safety analysis.

VARIABLE	PRE-TREATMENT	POST-TREATMENT
AST (n=1000)	34.07±29.59 U/l	36.15±31.35 U/l **
ALT (n=988)	25.67±14.93 U/l	27.61±25.07 U/l **
Total bilirrubin (N=966)	0.44±0.24 mg/dl	0.47±0.24 mg/dl **
Alkaline phosphatase (n=1000)	146.32±77.27 U/l	120.60±93.33 U/l **

** $p < 0.001$ vs pre-treatment values.

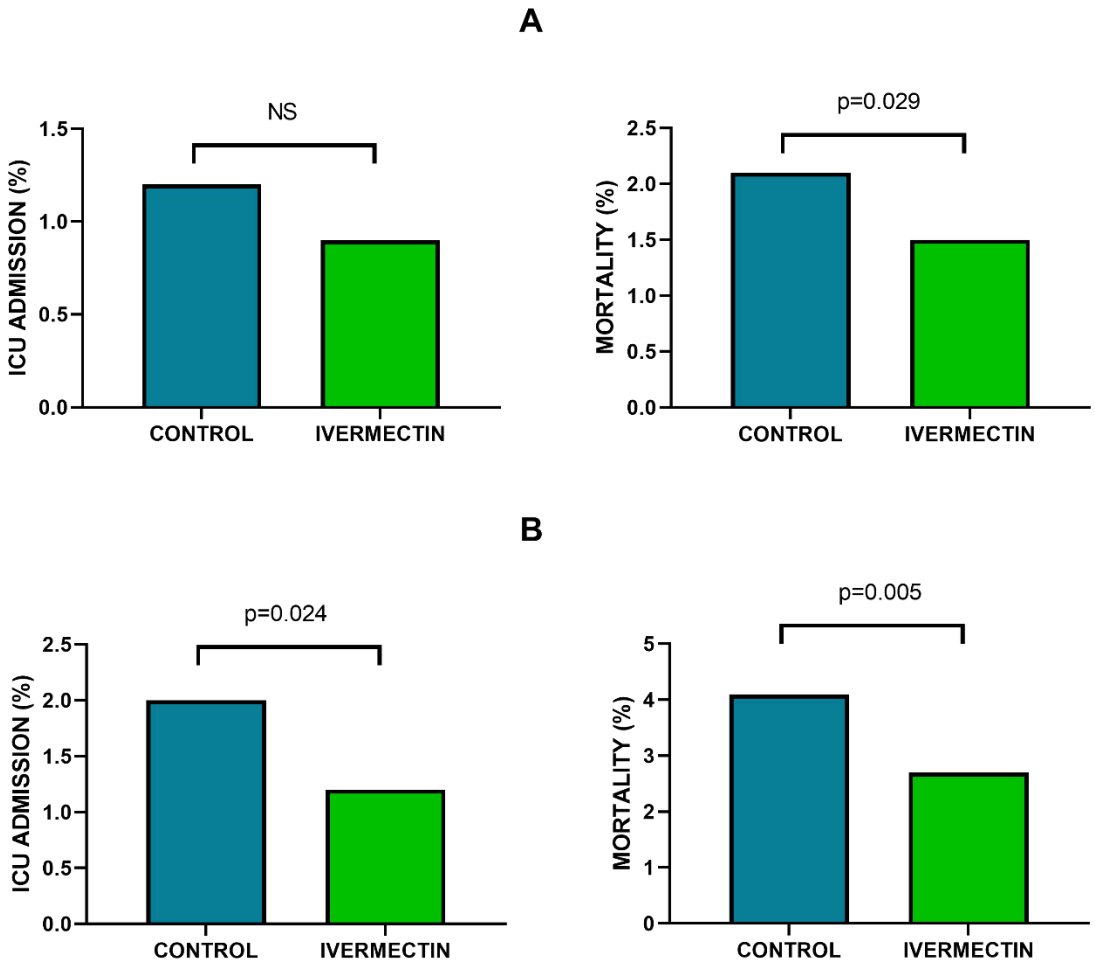
Program´s efficacy

In order to evaluate the program´s efficacy, the clinical evolution of subjects in the IVM-ITT analysis group (n= 3266) was compared with 17966 subjects that did not participate in the program (C group).

In the whole sample analysis, there was a non-significant tendency towards lower ICU admission in the IVM-ITT group compared with C (0.9% vs 1.2%, odds ratio 0.738) (95% CI 0.497-1.097, NNT 333, NS). Mortality rate was significantly lower in the IVM-ITT group (1.5% vs 2.1%) with an odds ratio of 0.720 (95% CI 0.535-0.969, NNT 172, (p=0.029) (Fig 2 A).

Regarding clinical evolution of subjects ≥ 40 year old (C n= 9022; IVM-ITT n=1851), ICU admission was significantly lower (1.2% vs 2.0, with an odds ratio of 0.608 (95% CI 0.393-0.940), NNT 128, (p=0.024). Mortality rate was significantly lower (2.7% vs 4.1%, with an odds ratio of 0.655 (95% CI 0.485-0.884), NNT 74, (p=0.005) (Fig 2 B).

Fig 2. ICU admission and mortality in IVM-ITT and C groups. Fig 2A: Analysis of all subjects ≥18 year-old (C n= 17966; IVM-ITT n=3266); Fig 2B: Analysis of all subjects ≥40 year-old (C n= 9022; IVM-ITT n=1851).



In the analysis of all non-immunized subjects (C: n= 16226; IVM: n=2936), there was a non-significant tendency towards lower ICU admission in the IVM-ITT group compared with C (0.7% VS 1.1%, odds ratio 0.639 (95% CI 0.406-1.005, NNT 250; p=0.051). Mortality rate was significantly lower in the IVM-ITT group (1.1% vs 1.7%, with an odds ratio of 0.628 (95% CI 0.434-0.907, NNT 158; p=0.012).

Regarding clinical evolution of non-immunized subjects ≥ 40 year old (C n=7463 and IVM-ITT n=1556), ICU admission was significantly lower (1.0% vs 2.1%, with an odds

ratio of 0.487 (95% CI 0.290-0.816, NNT 95;p=0.005). Mortality rate was significantly lower (2.1% vs 3.6%), with an odds ratio of 0.566 (95% CI 0.391-0.820, NNT 66;p=0.002).

A total of 2895 subjects in the IVM group had complete data regarding weight-based ivermectin dose information (of 3266 subjects assigned to IVM, 146 had missing data regarding the prescribed dose, and 335 had missing body weight data).

Mean IVM prescribed dose was 44.15±11.83 mg/day, mean IVM prescribed dose per kg of body weight was 0.54±0,09 mg/kg/day . Based on these values, two IVM groups were created: Low-dose IVM (with prescribed dose lower than 0.54 mg/kg/day, n=1157) and High-dose IVM (with a prescribed dose equal or higher than 0.54 mg/kg/day, n=1738). No significant differences were observed between groups regarding descriptive characteristics (age, gender, immunization status and comorbidities; data not shown).

In the whole sample analysis of High-dose IVM vs Low-dose IVM, there was a non-significant tendency towards lower ICU admission in the IVM-ITT group compared with C (0.6% vs 1.2%), with an odds ratio of 0.472 (95% CI 0.209-1.067; p=0.065). There were no significant differences in mortality rate (1.2 vs 1.6%) -odds ratio of 0.733 (95% CI 0.392-1.369;NS).

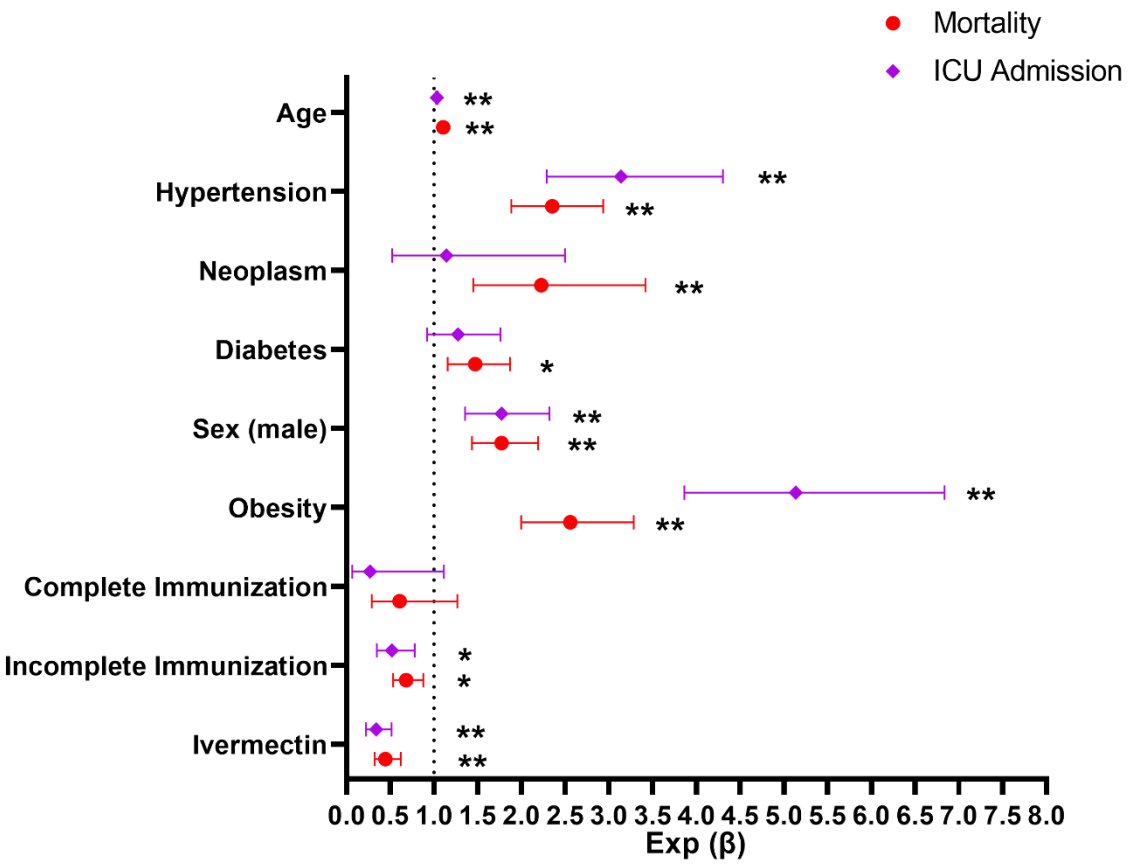
Regarding clinical evolution of subjects with an age of ≥ 40 year old (Low-dose IVM n=645 and High-dose IVM n=1016, there was a non significant tendency towards lower ICU admission in the High-dose IVM group (0.8% VS 1.7%)-with an odds ratio of 0.457 (95% CI 0.183-1.143;p=0.086). There were no significant differences in mortality rate (2.1% VS 2.9%) -odds ratio of 0.695 (95% CI 0.371-1.304;NS).

In the analysis of all subjects receiving High-dose IVM (n= 1738) vs C (n=17966), ICU admission was significantly lower in the High-dose IVM group compared with C (0.6% vs 1.2%), with an odds ratio of 0.494 (95% CI 0.261-0.934), NNT 172, (p=0.027). Similarly, mortality rate was lower in the High-dose IVM group compared with C (1.2 vs 2.1%) -odds ratio of 0.566 (95% CI 0.364-0.881, NNT 111;p=0.01).

Regarding clinical evolution of subjects with an age of ≥ 40 year old (C n=9212; IVM-ITT n=1016), ICU admission was significantly lower in the High-dose IVM group compared with C (0.8% vs 2.0%), with an odds ratio of 0.383 (95% CI 0.188-0.780, NNT 81;p=0.006). Similarly, mortality rate was lower in the High-dose IVM group compared with C (2.1 vs 4.1%) -odds ratio of 0.498 (95% CI 0.319-0.776, NNT 50; p=0.002).

The logistic regression analysis was performed after adjusting for sex, age, immunization status and comorbidities, and IVM treatment remained negatively associated with ICU admission rate and mortality rate (Fig 3).

Fig 3. ICU admission and mortality in patients receiving ivermectin within MEURI program vs Control after controlling for other variables by multiple logistic regression analysis. Age was considered as a continuous variable. Subjects were considered immunized after 14 days of their last vaccine dose. ** p<0.001; * p<0.01.



Discussion

This report of a monitored intervention program with ivermectin in COVID-19 patients provides observational data on a significant number of adult patients that through the incorporation of clinical and demographic data from a large number of patients from the same province and period but not participating in the program (control group) allowed a comparison and analysis of hard clinical endpoints as are admission to ICU

348 and death. This comparison provides results that suggest a significant positive clinical
349 impact of this intervention that, in the context of a lack of proven antiviral alternative
350 treatments for ambulatory patients against COVID-19, the safety, availability and
351 affordability of IVM and the growing concerns on vaccine efficacy against emerging
352 variants of SARS-CoV-2, deserves consideration as a potential tool for case
353 management.

354 The approach taken in the Province of La Pampa for the use of IVM through a MEURI
355 Program supported and leaded by the provincial Ministry of Health was based on
356 preliminary but inconclusive data on efficacy and a more solid reference base on the
357 safety of the drug, even at higher doses than those approved for other indications in
358 Argentina, as strongyloidiasis and scabies (21). With the incorporation of over three
359 thousand cases that completed treatment and follow up, including 1022 with clinical
360 laboratory monitoring, this intervention program contributes the largest analysis on the
361 safety of high dose ivermectin at a regimen of 600 µg/kg/day for 5 consecutive days.
362 This regimen was selected based on a proof-of-concept trial that identified a significant
363 antiviral activity against SARS-CoV-2 in a subgroup of participant who achieved high
364 IVM median plasma concentrations without any significant safety issues (16). In view
365 of that seemingly dose response antiviral effect, participants of our program were
366 advised to ingest the daily doses of IVM after a meal with adequate fat content in view
367 of the lipophilic nature of IVM (12,22) . The favorable safety profile of this regimen of
368 high dose IVM in a setting with conditions for high oral bioavailability confirms prior
369 communications with smaller sample sizes, on the safety of these regimens and allows
370 to focus in exploring the clinical efficacy of these regimens for a variety of clinical
371 indications for which IVM is in pre-clinical and clinical development as a repurposed
372 drug including COVID-19, dengue, trichuriasis and malaria. The finding of a single

case of clinically significant increase in liver enzymes, in an individual with baseline abnormal values highlights these clinical and laboratory findings and provides evidence for the design of simplified MEURI Programs, should they be considered appropriate.

The controversial findings around the efficacy of IVM in COVID-19 is currently preventing from making firm recommendations to clinicians. The situation is worsened by confusing messages through social and traditional media plus articles from peer-reviewed journals that are published and afterwards retracted as well as the uncontrolled use of medical and veterinary products by the population (23). Meta-analyses that included studies with a variety of regimens have reached different interpretations and conclusions, preventing the achievement of consistent findings (13,24–26). In this context, a MEURI Program appears as the mean to attempt monitored and controlled use of a treatment not approved for the indication but with preliminary results of adequate safety and potential efficacy. While the impossibility to perform clinical trials was not an absolute situation at the time of the design of our Program, the means and capacities of a provincial Ministry of Health were beyond the scope and resources available.

The role of IVM against SARS-CoV-2 is supported by the biologic plausibility based on in vitro and in vivo studies of mechanistic analyses and antiviral effects. Its proposed antiviral mechanism is thought to be mediated by its ability to inhibit the nuclear import of viral proteins mediated by IMPa/b1 heterodimer, and the promotion of defense mechanisms such as pyroptosis in infected epithelial cells, suggesting its possible role as a broad spectrum antiviral agent (27). These proposed effects might explain the antiviral activity against SARS-CoV-2 reported by our group and Biber et

al, in two small randomized controled trials (16,18). However, randomized clinical trials published on this topic have shown a lack of efficacy regarding clinical outcomes. Lopez Medina et al. reported on the failure to show a significant effect of 300 µg/kg of IVM for 5 consecutive days versus placebo in symptom´s resolution among 400 patients with mild disease recruited during the first week of COVID-19 in a single center in Colombia (14). This trial, that was originally designed to demonstrate improvement of 2 points in WHO Ordinary Scale but suffered from fewer than estimated events, included a population with a median age of 37 year-old and administered IVM on an empty stomach, as indicated by the manufacturer, which probably prevented from maximizing oral biovailability of this highly lipophilic drug. In the study from Vallejos et al. in Argentina (15), 501 patients with mild early COVID-19 infection were randomized to receive placebo or IVM for two consecutive days at up to 200 µg/kg (the currently approved dose for other indications). Neither hospitalization (primary outcome) nor other secondary outcomes including polymerase chain reaction test negativity and safety outcomes showed statistically significant differences between groups in this population with fewer events than estimated a priori and a mean age of 42 year-old (SD ± 15.5).

The seemingly contrasting efficacy results between our analysis of an intervention program versus the double-blind, placebo-controlled RCTs might not be discordant at closer look with reasons laying in several factors pertaining to IVM regimens, population size and outcomes; but potential bias in our results is another possibility to be considered and explored. The IVM regimen used in our program provided a higher total exposure to the drug through a higher dose per day, longer treatment (compared to the trial by Vallejos) and administration with food, which while equally safe might have allowed reaching drug levels at the relevant tissues above the threshold required

for an antiviral activity resulting in better clinical outcomes. This postulated dose effect has been observed in other studies and the high levels achieved in lungs and nasopharingeal mucosa were seen in an animal model (28). In terms of the populations included in the analyses, it is relevant to consider that both RCTs reached fewer primary endpoints than estimated for the sample size calculation; notwithstanding those potential limitations, which might have been affected by the age of the population recruited to those trials, neither adjustments nor secondary outcomes identified any significant clinical findings. Despite sound trial methodology, failure to demonstrate the effect of an intervention might reside in contextual elements as is the recruitment of subjects at very low risk of achieving the primary outcome regardless of the use of an intervention (29,30), as might have been the case in both RCTs as well as our observational analysis, which due to the significantly larger population size, was able to identify a statistically significant treatment effect in admission to ICU and death in restricting the analysis to those >40 year-old.

Statistically significant differences in observational studies should be viewed with caution since the clinical and public health relevance of those results might not be judged as relevant despite the statistics. Based on that, NNT ratios provide an indicator that could inform clinicians and policy makers on the value and convenience of this intervention. Effect size is another element to be considered in the evaluation of an intervention, since the ability of trials to rule-out the effect of an intervention grows in the required sample size in direct relationship to the decrease in the effect size, with direct implications in the feasibility of a clinical trial and the convenience of an intervention (31). In the present analysis, as expected, NNT related to ICU admission and mortality prevention differed significantly between subgroups, with a lower NNT in higher risk groups, as subjects older than 40 year-old.

As an observational intervention, our analyses are susceptible to bias, which constitute the most significant limitation of this report and a major concern in COVID-19 in view of the multitude of publications of studies and observations designed, run and published at unprecedented speed (32). The risk of confounding factors introducing bias in the comparison between groups cannot be completely ruled out, although several measures were taken to minimize its occurrence, like the verification of balanced age distribution, vaccination status and prevalence of comorbidities, with a special attention paid to current oncologic processes that could identify patients with terminal disease. In reference to it, in a sub-analysis that excluded individuals with current oncologic processes the differences between IVM and C groups remained significant (data not shown). Survivor bias was assessed and controlled in a sub-analysis (data not shown) through the exclusion from the analysis of all individuals in the control groups whose death occurred within the first 4 days since diagnosis, since those individuals were in all likelihood not offered the intervention, maintaining a significant association in favor of IVM in terms of mortality frequency in the higher risk groups (non-immunized subjects older than 40 year-old). In order to limit the mortality assessment to death related to COVID-19 in the C group, deaths were only considered for the analysis when occurring during the original hospital admission or within a month after discharge. Regarding the possible influence of differences in the prevalence of comorbidities between groups, it is important to highlight that, meanwhile most comorbidities were balanced between groups, a higher percentage of participants in the IVM group reported hypertension and obesity compared to C. Although these differences could be attributed to methodological differences in the identification of comorbidities, should they be real, describe a higher risk of disease progression and, consequently, would not explain the better outcomes observed in this group.

A per-protocol analysis including only individuals that completed the whole treatment, which could provide information of the full potential of the regimen was not performed since individuals on therapy that had their treatments interrupted at hospital admission were identified and given that situation, performing a per-protocol analysis would have given biased results that would wrongfully inflate the efficacy of the intervention.

One limitation of the present work is the absence of a detailed record of the refusal of patients to participate in the IVM program. Specifically, since the start of the program in January 2021, every physician in the province was authorized to offer this treatment to patients who met the inclusion criteria. However, the inclusion of patients was left to the discretion of the treating physician and to the acceptance by the patient. For this reason, the non-inclusion of patients in the IVM program could be due both to the refusal of patients to receive treatment after it was offered, to non-compliance with the inclusion criteria, or to the decision by the treating physician not to offer this treatment option. Unfortunately, no information is available to confirm the reason for not including each individual patient.

When looking at our findings in the context of the results and conclusions of rigorous RCTs, other observational clinical studies, virologic .and molecular biology studies for the evaluation of IVM in COVID-19, we conclude on the plausibility and potential clinical utility of IVM at higher doses than currently approved, optimizing its bioavailability but with a relatively moderate effect size in high-risk population groups. Without safety concerns at the doses used in our program, this report highlights IVM as an intervention that deserves a dispassionate, careful, public health-based consideration for the treatment of patients during present COVID-19 pandemic until

superior therapeutic alternatives become available and affordable, and highlights the importance of performing adequately powered RCTs in order to confirm our findings.

Acknowledgments

The authors would like to acknowledge the authorities of the Government of the Province of La Pampa, Argentina, for their support, and to the Ivermectin Monitored Intervention program collaborators: María Alejandra Ramallo; Andrea Barbero; Liliana Torres; Nadia Paola Andresco Soto; Lucía Martín; Analisa Beneitez; Marcela Natividad Millagueque; Carolina Andrea Spallina; Leandro Ceferino Deambrossio; Silvina Ramos Monso; Emiliano Renzo Beraudo; Raúl Javier Gavotti; Noelia del Valle Godino; Nora Ester Bertón; Carla Noelia Morales; Mariné del Valle Plizzo; Héctor Marsollier; María Estela Martin, Eugenia María Tabares, Lesly Godoy; Gerardo Minudri; Lourdes Bramante; Natalia Mendoza; Paloma De la Prida; María Soledad Arambarry; Silvia Lluch; Vanesa Garro; Santiago Arisi; Silvia Kredes; Gisela Bissio; Marcelo Allara; Juan Guerrero; Pablo Gutierrez; Pamela Astesano, Natalia Baez; Pamela Ulivetti; Mariano Quiroga; Romina Londero; Lorena Carasay; Diego Arroyo; Florencia Calo; Ruth Villagra; Marianela Barbero; Betina Larroque; Angihomar Hernandez; Maria Julia Luján; Enrique Quiroga; Silvia Romera; José Alfonso; Gastón Verna; Nancy Jouly; Gustavo López; Gabriela Barra; Astrid Pérez; Darío Bustos; Yanina Monarde, Dafne Rojas; Franco Balsa; Paola Cervellini, Ariel Soto; Silvia Soto; Cesar Sosa; Bibiana Widemman; Adolfo Barrionuevo; Cecilia Schneider; Adrián Díaz, Luisa Macayo; Ludmila Stern; Maximiliano Elias; Fernando Andreatta; Juleth Mercado: Horacio Ortiz; Oscar Mas; Marcelo Carrizo; Ludvina Luft; María Barrionuevo; Liliana Burgahrtd, Mariana Puglie; Jessica Puglie; María Naab; Carolina De los santos; Ruben Solett; Rocío Cerrada; Carolina Redel; Gloria Baigorria; Gustavo Topfanini; Norberto Rovera; María Morales; Nora Delfino; Amalia León; Alejandra Torres; Matías Muria; Clarisa Gómez; Jorge Jorja; Daniel Celi; Franco Saini, Natalia Vago; Patricia Gimenez; María Torres; Matías Altamira; María Juri, Noelia Rosa; Rita Guerbilli; Pamela Leguizamon; Jorge García; Mariela Torres; Daniela Anechi; Cristian González; Miguel Hermua; Silvina Furik; Noelia Baizero; Silvia Moran; Cecilia Abdala; Erika Yoshida; Anabella Penego; Agustina Abdala; Carla Barboza; Natalia Maison; Rosa Sepulveda; Alicia Cappozi; Vanesa Ferreyra; Yanina Verdugo; Daiana

Vanderberg; Dayana Iglesias; Maylen Aimetta; Pablo Sosa; Joaquín Cepeda; Gustavo Sosa; Lilia Schan; Laura Galarza.

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Funding

This work was supported by the Government of La Pampa, Argentina.

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FOI 26-3132 - Document 3

diagnostics

MDPI

Article

Changes in SpO2 on Room Air for 34 Severe COVID-19 Patients After Ivermectin-based Combination Treatment: 62% Normalization Within 24 hours

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Abstract: The emergence of COVID-19 in March 2020 challenged Zimbabwe to mount a response with limited medical facilities and therapeutic options. Based upon early clinical indications of efficacy for the macrocyclic lactone Ivermectin (IVM) against COVID-19, IVM-based combination treatments were deployed to treat this infection. Data were retrospectively analyzed for 34 severe, hypoxic COVID-19 patients, median age 56.5, who were provided this treatment at clinics or at home between August 2020 and May 2021, for whom SpO2 values were recorded all on room air. All but three of these 34 patients had significantly increased SpO2 values within 24 hours after the first IVM dose. The mean increase in SpO2 as a percentage of full normalization to SpO2=97 was 55.1% at +12 hours and 62.3% at +24 hours after start of treatment. These results parallel similar sharp, rapid increases in SpO2, all on room air, for 24 mostly severe COVID-19 patients in the USA (California) who were given an IVM-based combination treatment. All patients in these Zimbabwe and California critical series recovered. These rapid increases in SpO2 values after IVM treatment stand in sharp contrast to declines in SpO2 and associated pulmonary function following onset of moderate or severe COVID-19 symptoms under standard care.

Keywords: COVID-19, SARS-CoV-2, spike protein, ivermectin, doxycycline, SpO2

Citation: Stone, J.C.; Ndarukwa, P.; Scheim, D.E.; Dancis, B.M.; Dancis, J.; Gill, M.G.; Aldous, C. Changes in SpO2 on Room Air for 34 Severe COVID-19 Patients After Ivermectin-based Combination Treatment: 62% Normalization Within 24 hours. *Diagnostics* **2022**, *12*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Javier Fernández

Received: date
Accepted: date
Published: date

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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1. Introduction

Evaluating the efficacy of COVID-19 therapeutics is challenging since most patients typically recover; the worldwide cumulative case fatality rate was 2% as of September 2021 [1]. Thus, randomized clinical trial (RCT) results are important, yet interpretations, for example, of the more than 20 mostly positive RCT results for COVID-19 treatment with the drug ivermectin (IVM) [2-5] are controversial. Even for a drug such as remdesivir that was tested with several RCTs having large patient cohorts, opposite conclusions were drawn by the US FDA [6] and the World Health Organization [7] as to efficacy against COVID-19.

As a complement to positive RCT results for a COVID-19 therapeutic, a quantifiable determination of rapid, major improvement in pulmonary function, especially for severe COVID-19 patients, would further indicate efficacy and provide insights into that drug's underlying biological mechanism. The simplest indicator of lung function is blood oxygen saturation level, SpO2, measured with a pulse oximeter. However, the administration of supplemental oxygen to any COVID-19 patient would preclude meaningful pre-and post-treatment comparisons of SpO2 values. For this retrospective study conducted in

FOI 26-3132 - Document 3

Zimbabwe, ironically, challenges that constrained treatment capabilities also provided the opportunity to track changes in SpO2, all on room air, for 34 hypoxic, severe COVID-19 patients within 12 to 48 hours after beginning IVM-based combination treatment (see Figure 1).

Zimbabwe, a landlocked country in Southern Africa that shares a border with South Africa, had its first reported case of COVID-19 in March 2020 [8]. Eight cases and one death from COVID-19 followed in the same month [9]. COVID-19 wards were created at a general practice clinic by converting two staff rooms into a 4-bed ward and a storeroom into a 2-bed ward. Available equipment included several oxygen cylinders, an oxygen concentrator, six beds, and three monitors for SpO2 and blood flow parameters. The staff consisted of the lead author, another primary care physician, and either one or two nurses at different times, each on 12-hours shifts. During the initial months of the pandemic, in the absence of proven therapies and protocols, the standard of care evolved through early August 2020 to include corticosteroids, anticoagulants and antibiotics, as used elsewhere in the world during this period.

However, the efficacy of these treatments was found to be limited, and by the end of July 2020, several COVID-19 deaths were recorded in the country. Based upon reports of initial success using IVM for COVID-19 treatment from colleagues in Johannesburg, South Africa, the College of Primary Care Physicians of Zimbabwe (CPCPZ) adopted and included IVM in their COVID-19 treatment protocol from August 8, 2020, starting initially with a 10-12 mg stat dose. Treatment of COVID-19 with IVM continued after the first patients showed improved outcomes, with more rapid recoveries achieved at doses higher than the standard of 200 ug/kg as initially used. In August 2020, after it became apparent that IVM added to standard of care was significantly reducing the death rate, together with the hospital system being overwhelmed, CPCPZ physicians decided to treat COVID-19 patients where an IVM-based protocol could be administered, including at local general practice clinics which had nursing care and oxygen, and at some patients' homes with nursing support and oxygen supplementation as available.

1.1 IVM for COVID-19 treatment

The decision to include IVM in COVID-19 treatment protocols in Zimbabwe was made as the pandemic swept through that nation, overwhelming limited clinical care facilities, with no drug developed to treat COVID-19 being generally accepted as effective. A published case-controlled study of IVM treatment for COVID-19 conducted at four US hospitals [10] that had been initially released in a preprint in June 2020 found a 40% reduction in mortality among 173 patients treated with low dose IVM vs 107 case-matched controls (15% vs 25.2% deaths). Interest in IVM was supported by its Nobel prize-honored pedigree and its extensive use to treat a variety of human diseases in over 3.7 billion doses worldwide since 1987 [2,11,12]. Another favorable characteristic of this drug is its extraordinary record of safety, well tolerated at high doses [13,14], including in studies for COVID-19 treatment [15,16]. It is generally non-toxic even at doses far exceeding the therapeutic range [17,18]. Since August 2020, inpatient and outpatient treatments of COVID-19 with IVM have been applied across 25 countries [2], with more than 20 RCTs conducted for IVM treatment regimens [2-5].

A comprehensive review of clinical studies for IVM treatment of COVID-19 by the Nobel co-laureate for IVM, Dr Satoshi Omura, and colleagues, concluded that IVM yielded major reductions in mortality [2]. Two animal studies of IVM treatment at low human-equivalent doses, one for the SARS-CoV-2 virus in golden hamsters [19] and another for a related betacoronavirus (MHV-A59) in mice [20], found statistically significant treatment benefits. The indicated biological mechanism of IVM, competitive binding with SARS-CoV-2 spike protein [21], is likely non-epitope specific, possibly yielding full efficacy against emerging viral mutant strains.

The demonstrated safety of IVM at much higher than standard doses [13-16] allowed the latitude for dose escalation for IVM treatment of COVID-19 over time, and

FOI 26-3132 - Document 3

combination therapy centered around IVM plus doxycycline and zinc became the standard COVID-19 treatment protocol used by the CPCPZ. This application of multiple drugs against COVID-19 was based on Zimbabwe's experience with prior infectious diseases, for which early, aggressive use of multiple drugs has been a core treatment principle. The potential efficacy of these adjuncts was later supported by successful clinical trials results with treatments using IVM in combination with doxycycline [22] or with doxycycline and zinc [23]. This combination therapy for COVID-19 has been researched and advanced by Thomas Borody [5], who in 1990 published the first clinical trial of using a triple therapy of three inexpensive repurposed drugs for *H. pylor*i [24], the underlying bacterial cause of peptic ulcers. This triple therapy of repurposed drugs became the worldwide standard of care for peptic ulcers a decade later, after the patents for the palliative drugs Tagamet and Zantac expired, and the discovery of *H. pylor*i as the cause of peptic ulcers was honored with the Nobel Prize for Medicine in 2005 [5].

2. Materials and Methods

This study is a retrospective review of clinical data collected for 34 severe COVID-19 patients for whom SpO2 values were monitored all on room air during the course of their treatment, with therapeutic agents selected by their physicians to offer the greatest chances for clinical benefits and recovery. Clinical data were collected for additional patients in Zimbabwe for whom SpO2 values on room air were not recorded, thus not fitting the inclusion criteria of this analysis, with mortality and deterioration outcomes for that larger patient series having been previously reported [25].

2.1 Outcomes

Outcomes tracked were changes in SpO2 values from within one hour before treatment to 12, 24 and 48 hours after start of treatment (first dose of IVM), for 34 COVID-19 patients for whom a pre-treatment SpO2 value and at least one SpO2 value up to +48 hours after start of treatment were available, all obtained on room air.

2.2 Participants

The 34 COVID-19 patients of this study were from Harare, treated by CPCPZ physicians either at local clinics or at patients' homes.

2.3 Inclusion and Exclusion criteria

Patients selected for analysis were of age 18 or older and had treatment start dates between August 8, 2020, and May 31, 2021. Patients were required to have been found COVID-19 positive either by a PCR test or a clinical diagnosis made by criteria including exposure to a COVID-19 patient, hypoxia, lymphopenia, monocytosis, elevated LDH, elevated dimer, and/or radiology consistent with pulmonary abnormalities caused by the virus. Patients selected had an SpO2 value on intake of 51-93%, with all SpO2 values recorded and documented to have been obtained on room air (in almost every case because oxygen was not available), and at least one SpO2 value obtained within 48 hours after IVM administration. These patients thus fit the US National Institutes of Health's definition of severe COVID-19, a sufficient condition of which is an SpO2 value of 93% or below [26].

2.4 Treatment

All patients were administered an IVM-based combination therapy also including doxycycline and zinc plus selected other agents from the standard of care used prior to August 2020. These agents used in combination with IVM included corticosteroids, clopidogrel, aspirin, enoxaparin, a nebulized nano-silver preparation (applied for all patients), rivaroxaban, zinc sulfate, azithromycin, doxycycline, and in some cases, an IV antibiotic. Patients treated in a clinic were assessed by a nurse upon admittance, with blood drawn and PCR tests conducted as feasible given the patient's condition and with severe symptoms necessitating immediate treatment. For those patients who contacted a

FOI 26-3132 - Document 3

CPCPZ physician from home requesting treatment, an online questionnaire was first completed by the patient, after which, if COVID-19 was still a suspected diagnosis, a nurse visit to the home was conducted, with associated follow up per the procedures described for in-clinic patients.

Table 1. Patient age group, sex, and range of pre-treatment SpO2 values.

Age Group	Sex	Initial SpO2 (%) 66-84	85-89	90-93	Total
25-49					
	Female	2	2	1	5
	Male	2	2	2	6
	Total	**4**	**4**	**3**	**11**
50-59					
	Female	2	2	1	5
	Male	2	2	2	6
	Total	**4**	**4**	**3**	**11**
60-69					
	Female	0	1	1	2
	Male	0	4	2	6
	Total	**0**	**5**	**3**	**8**
70-79					
	Female	0	1	0	1
	Male	1	0	0	1
	Total	**1**	**1**	**0**	**2**
80+					
	Female	0	1	0	1
	Male	0	1	0	1
	Total	**0**	**2**	**0**	**2**
TOTAL		**9**	**16**	**9**	**34**

* Mean age=55; median age=56.5.

As evidence of IVM safety and tolerability accrued following its use beginning in August 2020, its stat dose of 10 mg as used for the earliest patients was increased on September 11, 2020, to 10-12 mg every four days for three doses. Subsequently, the dosage was further increased to 12 mg IVM on the day of admission and then on days 4 and 8 plus doxycycline (100mg b.i.d.) and zinc sulfate (60mg/day). The latter regimen was used up through December 2020, when the second pandemic wave emerged in Zimbabwe. At that time, additional evidence of safety and tolerability of this regimen supported further dose escalation to a standard IVM dose regimen of 12 mg daily for five consecutive days, with adjunct use of doxycycline and zinc sulfate continued at the doses noted. In some cases, for which this standard treatment regimen did not yield significant clinical gains within a few days, even higher doses of IVM were used, in some cases as high as 100 mg for a single dose. Transient adverse effects (AEs) such as blurred vision characteristic of high dose IVM often occurred at those dose levels, but no serious AEs associated with IVM were manifested in any patient.

2.5 Data collection

SpO2 values were tracked using monitors that continually displayed these plus readings for pulse rate and blood pressure and waveform images of blood pulses. For those patients treated at home, the intake nurse provided a pulse oximeter to the patient, unless the patient had one which the nurse deemed of reliable quality. The patient or a family member took SpO2 readings regularly, using the same oximeter as used for the pre-treatment reading. In most cases, these readings were taken daily, with much lower frequency after SpO2 values had risen significantly and the patient's clinical condition had correspondingly improved. Patients were instructed to message the nurse immediately if SpO2 ever decreased from a higher value to 93% or below or if any new clinical symptoms of concern developed.

FOI 26-3132 - Document 3

2.6 Analytical methods

For the 34-patient severe COVID-19 patients of this study, a measure of percent normalization toward a fully optimal SpO2 value of 97 was applied, which for pre-and post-treatment SpO2 values S_0 and S_1, is: $100*(S_1-S_0)/(97-S_0)$, capped at 100%. Pre- and post-treatment SpO2 values were plotted for all 34 patients using this percent normalization measure in Figure 1 and were also presented directly in Figure 2, Figure 3, and Table S1.

3. Results

Figures 1 and 2 and Table S1 show the progression of pre-treatment and post-treatment SpO2 values within 48 hours after the start of treatment (including first dose of IVM) for the 34 COVID-19 patients of this study, all of whom were treated with IVM, doxycycline and zinc. All of these patients recovered. SpO2 values are shown at pre-treatment, all recorded within one hour before the start of treatment and within 12, 24 and 48 hours after first dose of IVM. The SpO2 value shown for a given patient at time x is that for the latest post-treatment time ≤ x. (Thus, for example, for a patient having an SpO2 measurement at hour 17 as the only one in the first 48 hours after initial treatment, that would be used as their +24 and +48 hour values.)

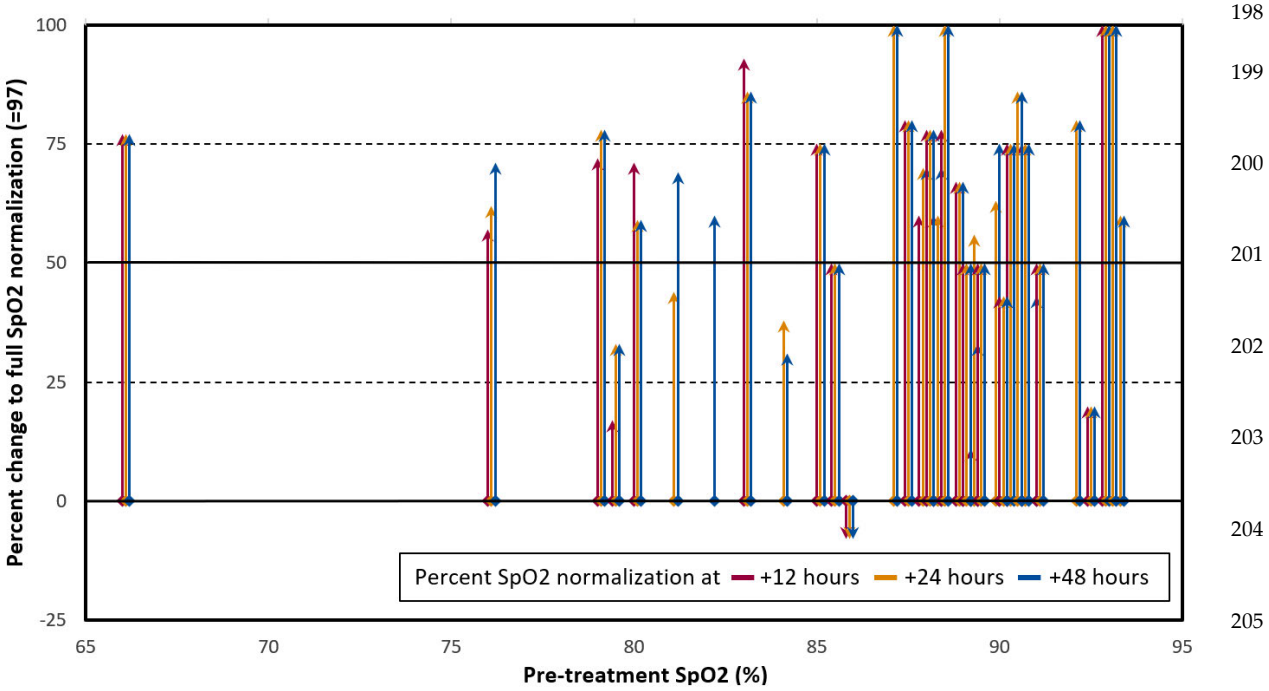


Figure 1. Changes in pre- to post-treatment SpO2 values, all on room air, for 34 severe COVID-19 patients treated with ivermectin (IVM), doxycycline and zinc, as percentages of optimal normalization to SpO2=97. Red, orange and blue lines represent, respectively, changes in SpO2 values at +12, +24 and +48 hours after first dose of IVM. Arrows were shifted to the right slightly (<1.5 x axis units) as needed for clarity. For pre-and post-treatment SpO2 values S_0 and S_1, the y axis is the percent of optimal normalization to SpO2=97, that is: $100*(S_1-S_0)/(97-S_0)$, capped at 100%. Mean (±SD) SpO2 changes (y values) were 55.1% ± 28.0% at +12 hours, 62.3% ± 26.3% at +24 hours and 64.3% ± 24.5% at +48 hours.

Figure 1 shows, for all 34 patients, SpO2 value changes from before IVM administration to post-treatment as percentages of full normalization to an optimal SpO2 value of 97 (95 is considered the minimum normal SpO2 value for a healthy child or adult by the US CDC [27]). Red, orange and blue lines show, respectively, SpO2 values at +12, +24 and +48 hours post-treatment. The mean (±SD) SpO2 changes as this specified percent of optimal normalization were 55.1% ± 28.0% at +12 hours, 62.3% ± 26.3% at +24 hours and 64.3% ± 24.5% at +48 hours. As shown in Figure 1, the bulk of the patients had 50% or

FOI 26-3132 - Document 3

more SpO2 normalization within 48 hours, while the sickest 3 (pre-treatment SpO2 ranging from 66 to 78%) improved by over 70%.

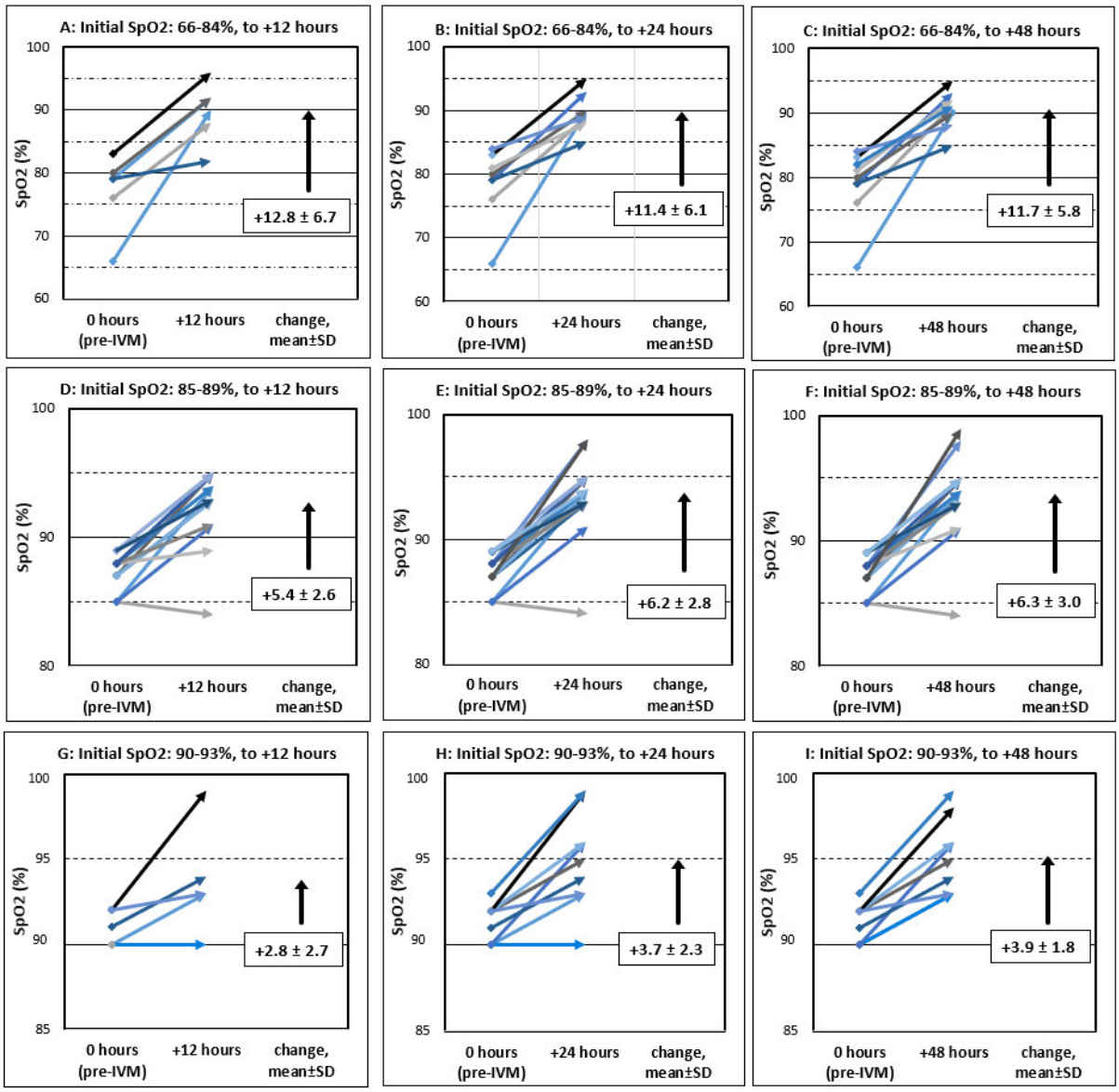


Figure 2. Pre- and post-treatment SpO2 values, all on room air, for 34 severe COVID-19 patients treated with IVM, doxycycline and zinc. Graphs A-I are in horizontal groupings by the range of pre-treatment SpO2 value and in vertical groupings by elapsed time after first dose of IVM. (The lowest SpO2 value in the stipulated range of 51-93% was 66%.) For these elapsed times of x=12, 24, and 48 hours, the SpO2 value shown for a given patient is at the latest post-treatment time ≤ x. All pre-treatment SpO2 values are from within one hour before the start of treatment.

Figure 2 shows all pre-and post-treatment SpO2 values grouped into nine graphs (A-I) by the range of pre-treatment SpO2 values and post-treatment times (after first IVM dose) of +12, +24 and +48 hours. As shown in Figure 2 and identically in Table 2, those patients with lowest, mid-range and highest pre-treatment SpO2 values had mean SpO2 increases at +12 hours of 12.8, 5.4 and 2.8, respectively (all SpO2 values in percentage units). Figure 3 shows all pre-and post-treatment SpO2 values throughout the entire observation period for each of the 34 patients. Note that these values were recorded less frequently after SpO2 had normalized.

FOI 26-3132 - Document 3

Table 2. Mean (± SD) changes in SpO2 values from pre- to post-treatment with IVM-based combination therapy.

	SpO2 (%)		
Pre-treatment	at +12 hours	at +24 hours	at +48 hours
66 - 84%	+12.8 ± 6.7	+11.4 ± 6.1	+11.7 ± 5.8
85 - 89%	+5.4 ± 2.6	+6.2 ± 2.8	+6.3 ± 3.0
90 - 93%	+2.8 ± 2.7	+3.7 ± 2.3	+3.9 ± 1.8

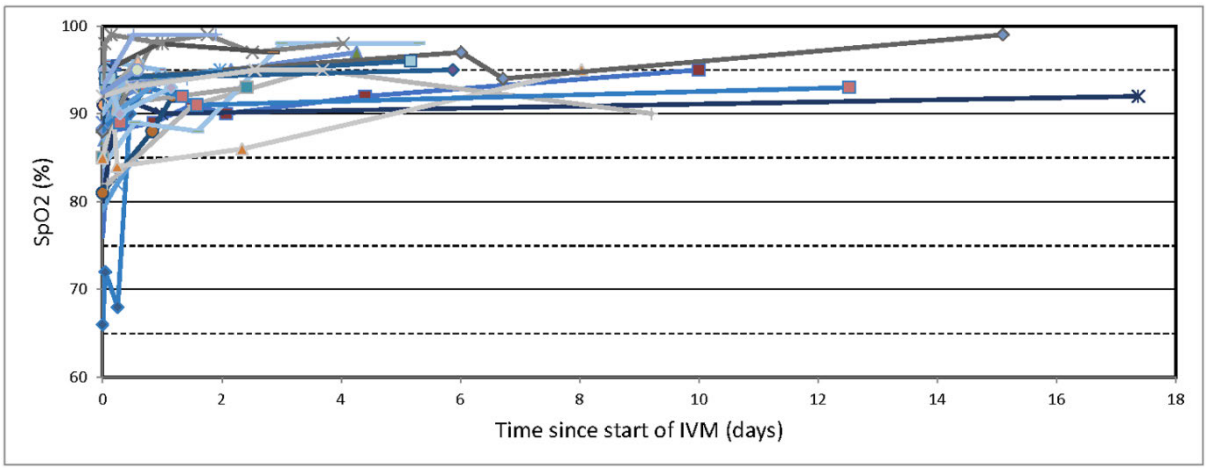


Figure 3. All SpO2 values (on room air) for the 34 severe COVID-19 patients of this study, all treated with IVM-based combination therapy including IVM, doxycycline and zinc.



Figure 4. Changes in SpO2 over 3 hours for a COVID-19 patient after administration of IVM. SpO2 values are shown (all on room air), with pulse rates (bpm) below those, at pre-treatment (A) and for 45 minutes (B), 90 minutes (C), and 3 hours (D) after first dose of IVM. This patient was diagnosed with COVID-19 by a positive result from a rapid antigen test and treated with combination therapy including IVM, doxycycline and zinc plus other oral and nebulized adjunct agents from among those itemized above.

Figure 4 shows successive SpO2 values for one patient who had a particularly rapid increase in these values after his first dose of IVM. This patient was a 25-year-old male, treated by a CPCPZ physician at a GP clinic without a supplemental oxygen capability. He received his first 12 mg IVM dose (repeated over the next four days) immediately after entering the clinic with respiratory distress and bilateral pneumonia indicated by stethoscopic examination. His COVID-19 diagnosis was confirmed by a positive result from a rapid antigen test. As shown, his SpO2 values increased from that recorded immediately before treatment (79%) to values at 45 minutes (87%), 90 minutes (92%) and

FOI 26-3132 - Document 3

3 hours (95%) post-treatment. He was discharged later that same day, and his home SpO2 readings then fluctuated between 92% and 95% over the next three days. By the fourth day after discharge, his SpO2 stabilized at 97%, his pulse dropped to 77 from prior values over 100, and he resumed working from home.

As shown in Figure 2, all but two of the 34 patients in this study had increases in SpO2 within the first 48 hours after first dose of IVM. As shown in Figure 3, this overall increase in SpO2 continued throughout the entire observation period. However, several studies of moderate and severe COVID-19 patients under standard care that track SpO2, pulmonary abnormalities, or both establish that for most patients, SpO2 decreases in tandem with an increase in the extent of pulmonary CT abnormalities from the day of onset of disease symptoms through the second week following [28-34]. Thus, the expected change in SpO2 within 48 hours after first IVM dose would be < 0, and one-tailed paired t-test calculations can assess whether SpO2 values increased significantly > 0 at each time period tracked.

Taking into account some missing post-treatment values (see Table S1), there are 25, 33 and 34 pairs of pre- and post-treatment SpO2 values at +12 hours, +24 and +48 hours, respectively. Applying these paired t-test calculations, the SpO2 increases were highly significant for each time period: t=6.28, p=8.5E-07 at +12 hours; t=8.42, p=6.36E-10 at +24 hours, and t=8.81, p=3.47E-10 at +48 hours.

4. Discussion

This study is a retrospective review of clinical data collected amid the challenges of providing treatment with limited facilities and resources to COVID-19 patients with severe disease. Under such conditions, it was not possible to obtain blood test values for all patients, including values for lymphocyte count, LDH, D-Dimer and CRP, which were thus not analyzed. On the other hand, the lack of availability of oxygen supplementation for many of the patients treated in Zimbabwe provided the rare opportunity to track changes in SpO2 values all recorded on room air before and after administration of IVM for this group of 34 severe COVID-19 patients, 74% of them presenting with SpO2 values below 90% and 29% presenting with SpO2 85% and below.

The mean (±SD) SpO2 normalization, as reported above, was 55.1% ± 28.0% at +12 hours, which rose to 62.3% ± 26.3% at +24 hours and then to 64.3% ± 24.5% at +48 hours after first IVM dose. Paired t-test calculations yield p<0.0000001 for each of the SpO2 increases, at +12, +24 or +48 hours, having occurred by chance had the IVM-based combination therapy applied had no clinical activity against COVID-19. Because the dearth of significant spontaneous improvements in SpO2 levels and respiratory function one day after a severe COVID-19 patient's presentation for medical care is a well-established norm for this disease per studies cited above, these probability values are significant and noteworthy.

This study's results, including the recoveries of all 34 patients, parallel those recently reported by Hazan et al., for which SpO2 values all on room air for 24 RT-qPCR confirmed COVID-19 patients were tracked before and +24 hours after combination treatment with IVM, doxycycline and zinc [35]. For the 19 severe COVID-19 patients in that series who had pre-treatment SpO2 values of 90% and below (minimum=77%) and had +24 hour post-treatment SpO2 values (see Table S2), the mean (±SD) SpO2 values rose from 86.7 ± 4.5 pre-treatment to 93.3 ± 2.6 at +24 hours after first IVM dose. Per our calculation of the percentage of normalization to SpO2=97, the mean (±SD) relative SpO2 increase at 24 hours post-treatment for these 19 patients was 65.2% ± 17.5%, which is close to the mean of 62.3% for the 34 patients in our study. The one-tailed paired t-test for these increased SpO2 values in these 19 patients yields t=9.34, p p<0.0000001, which as for our Zimbabwe study is highly significant.

The similar results for the COVID-19 patients in our Zimbabwe SpO2 series and the 24 patients of Hazan et al. suggest that the triple therapy of IVM, doxycycline and zinc provides efficacy regardless of which other adjunct agents were used, or may indicate that

FOI 26-3132 - Document 3

each study used different sets of adjuncts that further boosted the activity of this regimen to the degree of efficacy manifested in the clinical outcomes for each. One limitation of this study is that except for nebulized nano-silver, used for all patients at the start of the treatment regimen, other adjuncts were deployed on a case-by-case basis, per the patient's condition. Also, IVM dosages were increased during the study period based on the observations that no serious AEs were observed at higher doses, and higher doses appeared to be most effective for the patients with the most severe symptoms. A follow-up clinical study of IVM-based combination treatment of COVID-19 would benefit from a more structured specification and tracking of dosages and adjuncts used.

The distinct improvements in respiratory function for our study parallel results reported by Hazan et al. and provide a quantifiable demonstration of rapid clinical improvement in severe COVID-19 patients after IVM treatment. Such rapid improvements had been observed since the first major clinical trial in Florida, USA, of IVM treatment of COVID-19 [10]. The lead investigator of that clinical trial had observed that stabilization and then improvement in breathing function frequently occurred within 12-48 hours after IVM treatment, even for patients who had been deteriorating rapidly and had required supplemental oxygen at up to a 50% mixture [36]. That Florida study also reported a 40% reduction in mortality in the IVM-treated group compared with propensity-matched case controls. Likewise, for a larger series of 92 severe COVID-19 patients treated in Zimbabwe with IVM, doxycycline and zinc and other agents, the number of case fatalities (2) and deteriorations followed by recoveries (2) were much lower than predicted for a comparable set of patients using a well-regarded risk assessment model [25].

The SpO2 increases within a day after IVM treatment observed in this study and by Hazan et al. provide a distinct indication that IVM not only yields statistically significant clinical benefits in groups of patients as reported in most of the 20 RCTs for IVM treatment but provides rapid, directly observable resolution of pulmonary dysfunction as tracked by SpO2 values for COVID-19 patients. This finding offers clues as to the potential biological mechanism of IVM activity against SARS-CoV-2 since, for example, even an effective freeze on viral replication or rapid repair of damaged pulmonary alveoli would be unlikely to cause such rapid clinical improvements. One indicated biological mechanism of IVM activity, competitive binding with SARS-CoV-2 spike protein, as reviewed [21,37], may, through a reversal of virally induced hemagglutination, act quickly to increase pulmonary capillary flow and in turn account for normalization of blood oxygenation. An additional plausible mechanism of IVM activity is the activation of the cholinergic anti-inflammatory pathway under the control of the vagus nerve [38], which is regulated by acetylcholine and potentiated by the high-affinity binding of IVM (a positive allosteric modulator) to the alpha 7 cholinergic receptor a7nAChr [39] expressed on bronchial, vascular as well as to cytokine-producing cells (i.e., TNF, IL1 and IL6 secreting macrophages, lymphocytes and mast cells) [40].

For our study, rapid increases in SpO2 after start of IVM-based combination treatment occurred for all but two of these 34 patients and resulted in recoveries for all of them. No serious adverse effects (AEs) from IVM treatment were observed in any patient, although transient AEs such as blurred vision characteristic of higher-dose IVM administration were observed in some patients given doses as high as 100 mg. While comparative results using higher vs lower doses were not systematically tracked, the practice of increasing IVM doses for patients not initially responding to treatment had generally successful outcomes and supports the indication that higher doses provide greater efficacy.

5. Conclusions

Marked, rapid normalizations of blood oxygenation, $p<0.0000001$ (paired t-test), were obtained in each time period analyzed for the 34 severe COVID-19 patients in this study, all treated with IVM-based combination therapy. These results parallel similar

FOI 26-3132 - Document 3

rapid SpO2 increases reported by Hazan et al. for 19 COVID-19 patients having pre-treatment SpO2 ≤ 90, also at p<0.0000001, with all SpO2 values likewise on room air. For the 34 patients of our study, the mean increase in SpO2 value as a percentage of full normalization to SpO2=97 was 55.1% at +12 hours and 62.3% at +24 hours after first IVM dose, close to the mean SpO2 normalization of 65.2% at 24 hours after first IVM dose as calculated from SpO2 values reported by Hazan et al. These sharp, rapid increases in SpO2, accompanied by survivals of all patients in both the Zimbabwe and California critical series, stand in sharp contrast to the well-established typical decline in SpO2 during at least the first week after onset of moderate or severe COVID-19 symptoms. These SpO2 increases rapidly following first IVM dose establish a cause-and-effect clinical benefit for IVM-based combination treatment of this disease.

For the patients of this Zimbabwe study and the Hazan critical series, treatment at home or in clinics with basic facilities freed up hospital resources for other patients, and the treatment approach modeled in these studies could significantly relieve the pressure on overwhelmed health facilities. IVM is widely available worldwide, inexpensive, and one of the safest drugs in modern medicine, with its safety in "improving the health and wellbeing of millions" noted explicitly by the Nobel Committee in awarding its 2015 prize for the discovery of IVM [41]. These study results, therefore, support the extended deployment of IVM-based combination treatment for COVID-19, complementary to immunizations for prevention.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Changes in SpO2 for 34 COVID-19 patients treated with IVM, doxycycline and zinc.; Table S2: Changes in SpO2 for 19 COVID-19 patients treated with IVM, doxycycline and zinc as reported by Hazan et al., 2021.

Author Contributions: Conceptualization, J.C.S. and M.G.; methodology, J.C.S, M.G. and C.A.; software, B.M.D and D.E.S.; validation, B.M.D, J.D and D.E.S.; formal analysis, B.M.D, J.D and D.E.S.; writing—original draft preparation, J.C.S, D.E.S and C.A.; writing—review and editing, B.M.D, J.D. and D.E.S; project administration, J.C.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of The Medical Research Council of Zimbabwe (protocol code E293, June 23, 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data underlying the study cannot be made publicly available on an unrestricted basis due to ethical concerns about patient confidentiality. Data will be made available to qualified researchers on request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations:

The following abbreviations are used in this manuscript:

CPCPZ	Primary Care Physicians of Zimbabwe
COVID-19	coronavirus disease 2019
IVM	ivermectin
RCT	randomized clinical trial

FOI 26-3132 - Document 3

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From: s22
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Cc: Commercial Eyes Regulatory; s22
Subject: RE: Ivermectin s47 , doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy [SEC=OFFICIAL]
Date: Thursday, 25 August 2022 6:01:00 PM
Attachments: image004.png
image005.gif
image008.png
image009.png
image010.png
image011.png
image012.png
IRRS report on the LBS search strategy for Ziverdox2fin.pdf
Additional Clinical Guidance (July 2022) 27072022.docx
image002.png
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Hi s22

The TGA has reviewed the revised Nonclinical search strategy, provided in your email of 18 August 2022. Please find attached TGA comments on the revised Nonclinical search strategy.

TGA takes the opportunity to gently remind you of the other outstanding elements associated with this possible application, as provided in the document titled 'Additional Clinical Guidance (July 2022) 27072022', emailed to Commercial Eyes on 8 August 2022 (and copy attached for reference). Please let us know if you need any further clarification or assistance to addressing these elements prior to submitting an application on behalf of your client.

Regards,
s22

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From: s22 @commercialeyes.com.au>
Sent: Thursday, 18 August 2022 12:05 PM
To: s22 @health.gov.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>; s22 @commercialeyes.com.au>

Subject: RE: Ivermectin s47 , doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy [SEC=OFFICIAL]

REMINDER: Think before you click! This email originated from outside our organisation. Only click links or open attachments if you recognise the sender and know the content is safe.

Hi s22

Thank you for your feedback.

On behalf of our client Topelia Aust Ltd (Topelia) please find attached the new non-clinical search strategy document.

We look forward to receiving TGA feedback on the acceptability of the revised search strategy.

Thank you in advance.

Regards,
s22
Associate Consultant, Regulatory, Quality & Compliance

From: s22 @health.gov.au>
Sent: Monday, 8 August 2022 2:16 PM
To: s22 @commercialeyes.com.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Ivermectin s47 doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy [SEC=OFFICIAL]

CAUTION: This email is external and originated from outside Commercial Eyes. Do not click on links or open attachments unless you recognise the sender and sender's email address and know the content is safe.

Hi s22

Thanks for your patience.

Please find attached TGA feedback on your Literature based search strategy for Ziverdox.

Kind regards s22

Strategic Lead – COVID-19 vaccine and treatment regulation

Director – Application and Advisory Management Section

Prescription Medicines Authorisation Branch, TGA
Australian Government, Department of Health and Aged Care
s22
PO Box 100, Woden ACT 2606, Australia



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From: s22 @commercialeyes.com.au>
Sent: Thursday, 4 August 2022 1:18 PM
To: s22 @health.gov.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Ivermectin s47 doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy [SEC=OFFICIAL]

REMINDER: Think before you click! This email originated from outside our organisation. Only click links or open attachments if you recognise the sender and know the content is safe.

Hi s22

Many thanks for providing an update so promptly; much appreciated.

Regards,
s22

Innovative Therapeutics Manager, Regulatory, Quality & Compliance



From: s22 @health.gov.au>
Sent: Thursday, August 4, 2022 1:09 PM
To: s22 @commercialeyes.com.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>

Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Ivermectin s47 doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy [SEC=OFFICIAL]

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Hi s22 ,

My apologies for the delay in providing a response. The TGA is aiming to provide feedback on the LBS search strategy in the next fortnight.

Regards,
s22

Assistant Director - COVID-19 vaccine and treatment regulation
Case Manager - COVID-19 submissions
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
s22
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Wednesday, 3 August 2022 2:43 PM
To: s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Ivermectin s47 doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy [SEC=OFFICIAL]

REMINDER: Think before you click! This email originated from outside our organisation. Only click links or open attachments if you recognise the sender and know the content is safe.

Hi s22

On behalf of our client Topelia, may I please politely enquire into the progress of the LBS search strategy review for the proposed triple therapy Ziverdox?

CEPL and Topelia are more than happy to arrange a call to discuss any elements of the proposed LBS strategy at a time that suits.

Thank you in advance.

Regards,
s22

s22
Innovative Therapeutics Manager, Regulatory, Quality & Compliance

---

From: s22
Sent: Tuesday, July 19, 2022 5:22 PM
To: s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Ivermectin s47 , doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy [SEC=OFFICIAL]

Hi s22

Many thanks for advising; on behalf of our client Topelia, we appreciate the prompt reply.

Regards,
s22

s22
Innovative Therapeutics Manager, Regulatory, Quality & Compliance

---

From: s22 @health.gov.au>
Sent: Tuesday, July 19, 2022 4:53 PM
To: Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>; s22 @commercialeyes.com.au>; Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Ivermectin s47 , doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy [SEC=OFFICIAL]

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Hi s22

Thank you for your follow-up email.

Yes. The Literature based submission search strategy is under active consideration by the TGA clinicians, and TGA is aiming to provide Topelia feedback within the next 2 weeks.

Kind regards s22

Strategic Lead – COVID-19 vaccine and treatment regulation
Director – Application and Advisory Management Section

Prescription Medicines Authorisation Branch, TGA
Australian Government, Department of Health and Aged Care
s22
PO Box 100, Woden ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Tuesday, 19 July 2022 10:49 AM
To: Streamlined Submission <streamlinedsubmission@health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Ivermectin s47 , doxycycline s47 and s47 tablets - Request for advice on LBS search strategy

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Dear Sir/Madam,

On behalf of our client Topelia, may I please politely enquire into the progress of the below evaluation? Could you please advise the LBS Search Strategy is under evaluation by the appropriate clinical stream?

Many thanks in advance for any update that may be available.

Regards,
s22

Innovative Therapeutics Manager, Regulatory, Quality & Compliance

---

From: s22
Sent: Friday, July 8, 2022 2:47 PM
To: Streamlined Submission <streamlinedsubmission@health.gov.au>; streamlinedsubmission@tga.gov.au
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: Ivermectin s47 , doxycycline s47 and zinc s47 tablets - Request for advice on LBS search strategy

Dear Sir/Madam,

On behalf of our client Topelia Aust Ltd (Topelia), CEPL wish to seek TGA's feedback on the search strategy for a literature based submission, to support the Category 1 submission of a s47 comprising of ivermectin s47 , doxycycline s47 and zinc s47 s47 for the following indication:

- Ziverdox is indicated for the treatment of mild to moderate symptomatic patients diagnosed with COVID-19 caused by SARS-CoV-2 virus.

Please find further details about the submission and the proposed search strategy attached.

We look forward to receiving TGA feedback on the acceptability of the proposed search strategy.

Thank you in advance.

Regards,
s22

Innovative Therapeutics Manager, Regulatory, Quality & Compliance
Level 20, 15 William Street
Melbourne VIC 3000

Mailing Address:
PO BOX 620 COLLINS STREET WEST VIC 8007
s22
www.commercialeyes.com.au

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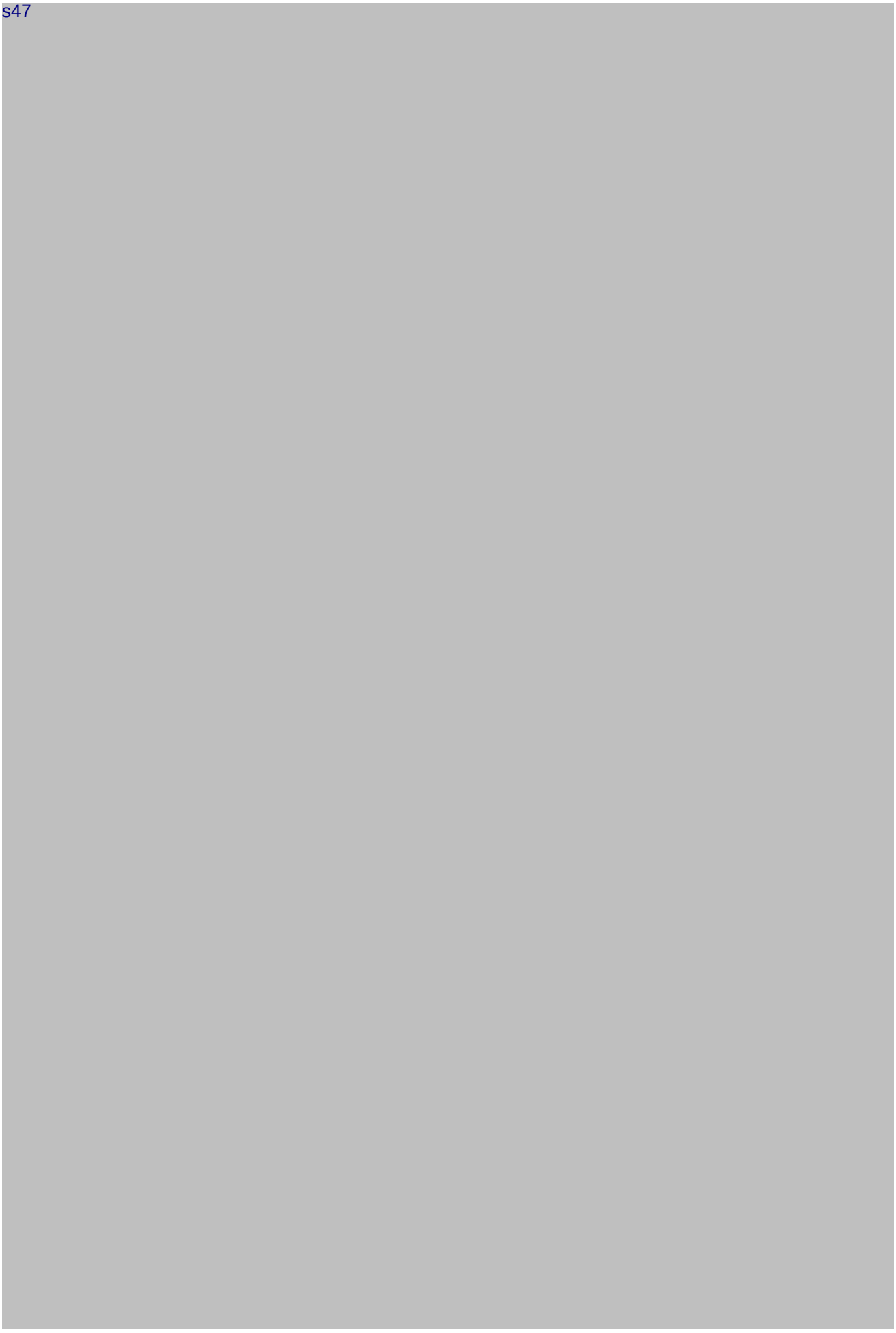
Proposed product: s47 ('ZIVERDOX')
Sponsor: Topelia Ltd
Agent: Commercial Eyes Pty Ltd
Proposed indication: *Ziverdox is indicated for the treatment of mild to moderate symptomatic patients diagnosed with COVID-19 caused by SARS-CoV-2 virus.*

The TGA reiterates the advice provided previously to s22 (email with 3 attached documents sent by TGA (Grant Pegg, dated 10 September 2021). The 3 documents that were previously provided:

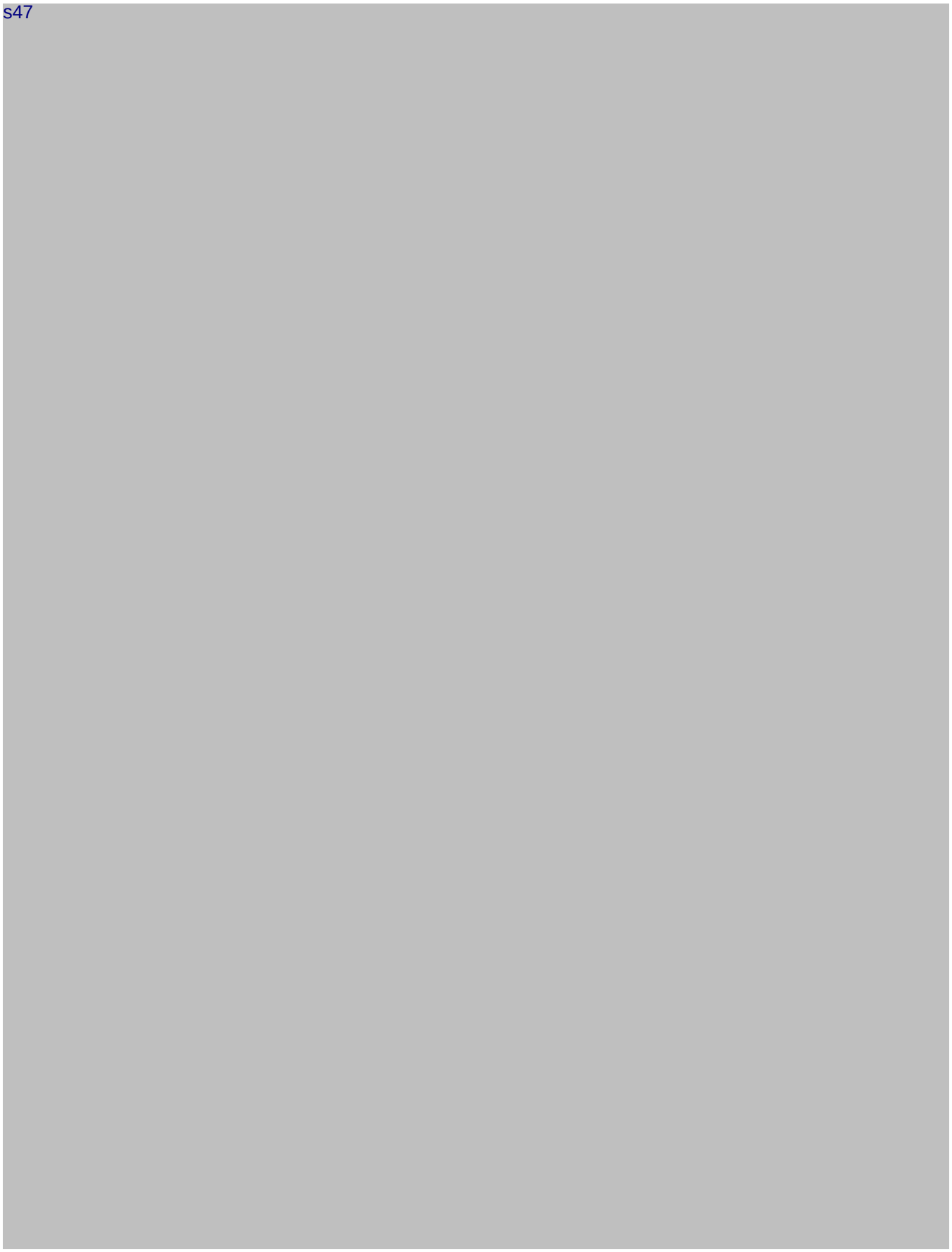
- Relevant guidelines for consideration
- Comments on the Clinical study synopsis
- Considerations when applying to register medicines

Following review of your background briefing document (provided to the TGA on 25 March 2022), the TGA provides the following advice s47

s47


s47


s47


s47

From: s22 ; Streamlined Submission
Cc: "Commercial Eyes Regulatory"; 'S22
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd; Email 1 of 4 [SEC=OFFICIAL]
Date: Thursday, 6 April 2023 1:14:00 PM
Attachments: image001.png
image002.png
image003.jpg
image004.png
image005.png
image006.gif

Hi s22

The TGA has screened the updated s47 justification for the proposed ivermectin/doxycycline/zinc citrate (ZIVERDOX) product.

The scope of the additional references and updated s47 justification do not appear to have addressed the data gaps previously noted by the TGA.

The TGA notes there are some new references provided, however these appear to be not directly relevant and/or are of lower quality to support the proposed claims. In addition, there a number of recent, higher quality studies that have not been referenced (for example: JAMA 2023, 329(11), 888-897. doi:10.1001/jama.2023.1650); an application should include a balanced view of available evidence.

Please note that the TGA does not typically provide advice on the clinical study design.

The Nonclinical area has also noted that the main nonclinical issue would be the lack of pharmacology and most importantly, toxicology studies with the combination given the limited clinical experience/data with the combination. No nonclinical pharmacology studies were noted in the justification to support the sponsor's claim of synergistic antiviral activity. There are no toxicology studies with the combination to assess potential toxicological interactions between the 3 ingredients.

Regards,
s22

Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
T: s22 | E: s22 @health.gov.au
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22
Sent: Friday, 24 March 2023 3:52 PM
To: s22 @commercialeyes.com.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: 'Commercial Eyes Regulatory' <regulatory@commercialeyes.com.au>; s22 @commercialeyes.com.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd; Email 1 of 4 [SEC=OFFICIAL]

Hi s22 ,

As noted in my email below, the TGA has received all 6 emails and attachments for Topelia's proposed product Ziverdox.
TGA is aiming to provide a response by mid-April.

Regards,
s22

Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
T: s22 | E: s22 @health.gov.au
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22
Sent: Wednesday, 22 March 2023 6:00 PM
To: s22 @commercialeyes.com.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>; s22 @commercialeyes.com.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd; Email 1 of 4 [SEC=OFFICIAL]

Hi s22 ,

Thank you. All 6 emails and attachments received.

Regards,

s22

Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
T: s22 | E: s22 @health.gov.au
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Wednesday, 22 March 2023 5:37 PM
To: s22 @health.gov.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>; s22 @commercialeyes.com.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd; Email 1 of 4 [SEC=OFFICIAL]

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Dear s22

Thank you for confirming receipt.

Please note additional emails (2, 3, 4, 5 and 6) were sent today due to further size restrictions.

Please confirm these were received.

Kind regards,
s22
Associate Consultant, Regulatory, Quality & Compliance

From: s22 @health.gov.au>
Sent: Wednesday, March 22, 2023 5:12 PM
To: s22 @commercialeyes.com.au>; s22 @health.gov.au>; Streamlined Submission

<streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>; s22 @commercialeyes.com.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd; Email 1 of 4 [SEC=OFFICIAL]

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Hi s22 ,

We have received 'Email 1 of 4'.
Grateful if you can please indicate if Emails 2, 3, and 4 were also sent today, as we do not appear to have received them?

Regards,
s22

Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
T: s22 | E: s22 @health.gov.au
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Wednesday, 22 March 2023 3:53 PM
To: s22 @health.gov.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>; s22 @commercialeyes.com.au>
Subject: Justification for s47 - Ziverdox, Topelia Aust Ltd; Email 1 of 4

REMINDER: Think before you click! This email originated from outside our organisation. Only click links or open attachments if you recognise the sender and know the content is safe.

Dear s22

On behalf of our client, Topelia Aust Ltd (Topelia), please find attached a response to the TGA justification review dated 31 October 2022.

The s47 justification response is accompanied by a full reference list for ease of review.

Please note due to file size, the remaining attachments will be provided across other emails. The email will be identified in the Subject line as Justification for s47 - Ziverdox, Topelia Aust Ltd; Email X of 4.

Topelia Aust Ltd are welcoming of a meeting to discuss TGAs thoughts and next steps for the s47 .

Kind regards,
s22
Associate Consultant, Regulatory, Quality & Compliance

From: s22
Sent: Monday, November 7, 2022 9:39 AM
To: s22 @health.gov.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd [SEC=OFFICIAL]

Hi s22 ,

Thank you and apologies for the delay, I was just in the process of replying. CEPL has received and on behalf of our client, Topelia, are preparing a response.

Regards,
s22

Innovative Therapeutics Manager,Regulatory, Quality & Compliance

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From: s22 @health.gov.au>
Sent: Monday, November 7, 2022 9:22 AM
To: s22 @commercialeyes.com.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd [SEC=OFFICIAL]

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Hi s22 ,

Just following-up if Commercial Eyes had received the TGA's email below of last Monday (31 Oct 2022), with the pdf attachment?

Regards,
s22

Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
T: s22 | E: s22 @health.gov.au
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22
Sent: Monday, 31 October 2022 4:05 PM
To: s22 @commercialeyes.com.au>; s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd [SEC=OFFICIAL]

Hi s22 ,

Further to the elements raised in TGA's email of 26 September, please find attached the TGA's initial review of the s47 justification for Ziverdox.
Note that the TGA would also take into consideration additional clinical studies that come to

hand (e.g. recent study published in JAMA last week).

Regards,
s22

Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
T: s22 | E: s22 @health.gov.au
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Friday, 30 September 2022 11:14 AM
To: s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>; s22 @health.gov.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd [SEC=OFFICIAL]

REMINDER: Think before you click! This email originated from outside our organisation. Only click links or open attachments if you recognise the sender and know the content is safe.

Good morning s22 ,

Thank you very much for providing the initial feedback on the s47 for Ziverdox.

A response on behalf of our client, Topelia, will be provided shortly.

Regards,
s22

Innovative Therapeutics Manager, Regulatory, Quality & Compliance

---

From: s22 @health.gov.au>
Sent: Monday, September 26, 2022 12:03 PM

To: s22 @commercialeyes.com.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>; s22 @health.gov.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd [SEC=OFFICIAL]

CAUTION: This email is external and originated from outside Commercial Eyes. Do not click on links or open attachments unless you recognise the sender and sender's email address and know the content is safe.

Good afternoon s22

Thanks for providing your justification for your s47 for this potential application.

TGA has completed an administration screen of the proposed justification. Grateful if you can please clarify a couple components of the proposed justification before the TGA continues its review. Please confirm,

1. the scope of the justification, specifically the date range of the literature references that were included in your s47 justification
2. both supporting and non-supporting literature articles were included in the development of the justification.
3. the literature articles that were referenced in the s47
4. a scientific rationale has been provided for proposed dosage of each component in the s47 based on the breadth of supporting and non-supporting literature studies using a weight of scientific evidence approach. Where non-supporting studies have been included, the justification has considered these articles and provided robust scientific reasoning as to why they were considered not relevant to the proposed s47 .

Once the TGA has received clarification of the above, the TGA can then provide you further details regarding next steps to support this potential application. Please feel free to reach out if you have any questions.

Kind regards s22

Director – Application and Advisory Management Section

Prescription Medicines Authorisation Branch, TGA
Australian Government, Department of Health and Aged Care

T: s22 | M: s22 | E: s22 @health.gov.au
PO Box 100, Woden ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Tuesday, 20 September 2022 11:00 AM
To: Streamlined Submission <streamlinedsubmission@health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>; s22 @health.gov.au>
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd [SEC=OFFICIAL]

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Hi s22 ,
Many thanks for advising; CEPL will advise our client.
Regards,
s22
Innovative Therapeutics Manager, Regulatory, Quality & Compliance

---

From: s22 **On Behalf Of** Streamlined Submission
Sent: Tuesday, September 20, 2022 10:21 AM
To: s22 ; Streamlined Submission
Cc: Commercial Eyes Regulatory ; s22
Subject: RE: Justification for s47 - Ziverdox, Topelia Aust Ltd [SEC=OFFICIAL]

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Hi s22 ,
Thank you for your email with the proposed justification for a proposed s47 medicine (Ziverdox).
The TGA will consider the justification, and aim to provide a response in the next 2-3 weeks.
Regards,
s22

Streamlined Submission Inbox - Application and Advisory Management Section

Medicines Regulation Division | Therapeutic Goods Administration
Australian Government, Department of Health and Aged Care
T: s22 | E: streamlinedsubmission@health.gov.au
PO Box 100, Woden ACT 2606, Australia

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Subject: Justification for s47 - Ziverdox, Topelia Aust Ltd

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Dear Streamlined Submissions,

On behalf of our client, Topelia Aust Ltd (Topelia), CEPL hereby submit a justification for a proposed s47, Ziverdox for consideration.

Ziverdox is a proposed s47 comprising of ivermectin s47 doxycycline s47 and zinc s47 for the following indication:

- *Ziverdox is indicated for the treatment of mild to moderate symptomatic patients diagnosed with COVID-19 caused by SARS-CoV-2 virus.*

The s47 justification is accompanied by a full reference list for ease of review.

CEPL look forward to receiving TGA's feedback on the proposed fixed dose combination therapy.

Thank you in advance.

Regards,

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FOI 26-3132 - Document 5

Pediatric Pulmonology 43:281–287 (2008)

Effect of Zinc Supplementation on Respiratory Tract Infections in Children With Cystic Fibrosis

I. Abdulhamid, MD,[1]* F.W.J. Beck, PhD,[2] S. Millard, MD,[3] X. Chen, MD, PhD,[1] and A. Prasad, MD, PhD[2]

Summary. Zinc (Zn) has significant anti-oxidant and anti-inflammatory activity. Zn deficiency can occur in subsets of patients with cystic fibrosis (CF) especially those with malabsorption and impaired growth. Although supplemental Zn has significantly reduced infections in various disorders, its efficacy has not been thoroughly investigated in CF. We performed a double blind placebo controlled pilot study to investigate the effect of daily 30 mg elemental Zn for 1 year on the rate of respiratory tract infections (RTIs), use of antibiotics and plasma cytokines in 26 children with CF (ages 7–18 years). Plasma Zn, Cu, inflammatory cytokines and ex vivo generation of IL-2 were measured at baseline and at the end of the study. The number of days of oral antibiotics was lower in Zn treated patients compared to placebo ($P = 0.05$). However, compared to placebo, the effect of Zn was greater in patients who exhibited low plasma Zn at baseline ($P = 0.02$) than those who had plasma Zn levels identical to normal subjects ($P = 0.55$). Zn supplementation was marginally effective in reducing percentage increase in plasma IL-6 and IL-8 while increasing the percentage change in ex vivo generation of IL-2 in isolated mononuclear cell. In conclusion, oral intake of 30 mg/day of Zn reduced the number of days of oral antibiotics used to treat RTIs in children with CF. A higher daily Zn dose may be needed to decrease RTIs and modify immune responses. **Pediatr Pulmonol. 2008; 43:281–287.** © 2008 Wiley-Liss, Inc.

Key words: cystic fibrosis; respiratory tract infections; zinc; cytokines.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by genetic mutations that code for cyclic AMP-activated chloride channel, the CF transmembrane regulator (CFTR).[1–3] The disease affects many organ systems, particularly the respiratory and gastrointestinal tracts. Due to pancreatic insufficiency, malabsorption of essential fatty acids, fat-soluble vitamins, and various trace elements, including zinc (Zn), can occur in subjects with CF.[4,5] The airways in patients with CF are uniquely susceptible to chronic infections with various bacterial agents, especially Gram negative organisms such as *pseudomonas aeroginosa*.[6] This infection causes the release of inflammatory cytokines, proteolytic enzymes, and oxygen radicals which leads to bronchiectasis, increased dead space, hypoxia, and hypercapnea.[7,8]

Approximately 90% of patients die of respiratory failure as a result of chronic bacterial infections, Treatment of CF lung disease is aimed at reducing airway obstruction, treating recurrent infections, improving airway clearance, and maintaining optimal nutritional status.[9–11]

Zn is an integral part of the structure and function of many biological enzymes and a regulator of ion transporters relevant to pulmonary function and disease in CF and other pulmonary disorders.[11–13] Zn is also known to exhibit powerful anti-oxidant activity in several organ systems including the lungs.[11,13] For example, Zn deficient rats developed significant lung toxicity 1 day after exposure to high oxygen concentrations and Zn replenishment prevented the hyperoxia lung damage in previously Zn deficient animals.[14]

Zn supplementation to non-CF infants and pre-school children resulted in reduced incidence of acute lower respiratory infections.[15] Zn supplementation lead to the reduction of plasma concentration of inflammatory cytokines, and oxidative stress markers in the absence of

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Grant sponsor: Children's Research Center of Michigan at CHM.

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Received 29 May 2007; Revised 10 November 2007; Accepted 13 November 2007.

DOI 10.1002/ppul.20771
Published online in Wiley InterScience (www.interscience.wiley.com).

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infection,[16] and decreased the duration of colds in healthy young adults.[17] It also lowered the incidence of infection and inflammatory cytokines levels in healthy elderly subjects.[18]

Using plasma and red cell Zn concentrations to reflect Zn status in CF has produced conflicting results as not all CF patients appear to exhibit low Zn status.[19] Administration of supplemental Zn to CF patients has also been non-conclusive for in one study, Zn therapy did not result in improvement of clinical status, growth velocity or pulmonary function.[20] Another study showed an association between low Zn status and impaired IL-2 activity, NK activity and thymulin activity in CF subjects, but there was no investigation as whether these parameters would be corrected following Zn supplementation.[21]

With these premises we initiated a pilot study to determine whether Zn supplementation administered to CF patients during a year long double blinded placebo controlled trial would reduce the requirement for oral antibiotics necessary during periods of active infection. We also assessed whether changes in selected plasma inflammatory cytokines and the ex vivo generation of IL-2 would be altered by oral Zn therapy. And lastly, we investigated the hypothesis that a response to Zn supplementation would be different in subjects with low plasma Zn levels compared to those expressing normal plasma Zn levels.

MATERIALS AND METHODS

Subjects and Controls

For this double blind placebo controlled study, a total of 26 children with CF exhibiting mild to moderate lung disease groups A and B were recruited from the Pediatric Pulmonary Division, Carman and Ann Adams Department of Pediatrics, Children's Hospital of Michigan (CHM), and the Pediatric Pulmonary Division, DeVos Children's Hospital, Grand Rapids, Michigan. Subject's ages ranged from 7 to 18 years old. Exclusion criteria for the study included presence of acute severe infection at the time of enrollment, renal disease, severe hepatic disease, gall bladder disease, sickle cell disease (SCD), use of oral immunosuppressive drugs (steroids and non-steroid anti-inflammatory drugs), diuretics and Zn supplements. The study was approved by the Human Investigative Committees in both institutions. Informed and signed consent was obtained from parents or legal guardians.

Study Groups

Without respect to prior Zn status, CF subjects were randomly assigned to Group A ($n = 13$) received a daily dose of 30 mg of elemental Zn as Zn gluconate (15 mg/capsule) and CF subjects assigned to Group B ($n = 13$) received placebo for 12 consecutive months. Zn gluconate and placebo preparations were capsules generously provided by Labcatal, Montrouge, France for use in this study. Both groups were given identical daily Zn-free and mineral-free multivitamin supplements for the same period (Vitamax® from SK Labs, Chesterfield, MO) supplied by the CF services Pharmacy. Patients in groups A and B were followed every 3 months and as needed in their CF clinics for routine CF care. At each visit, the patient's interval medical history, height and weight, number of hospitalizations, the use of oral and intravenous antibiotics, pulmonary function test, and physical examination findings were recorded.

Respiratory tract infections (RTIs) was defined when the patient developed more than four of the following signs or symptoms: change in sputum; new or increased hemoptysis; increased cough; increased dyspnea; malaise, fatigue, or lethargy; temperature above 38°C; anorexia or weight loss; sinus pain or tenderness; change in nasal or sinus discharge; change in physical examination of the chest; decrease in pulmonary function from a previously recorded value; or radiographic changes indicative of pulmonary infection.

Choice and duration of oral or parenteral antibiotics used for treatment of RTIs were left to the discretion of the children's treating physicians.

Ten milliliter of heparinized whole blood was collected at baseline and at the end of the study for determination of plasma Zn, Cu and the inflammatory cytokines, TNF-α IL-1β IL-6 IL-8, soluble IL-1 receptor antagonist (sIL-1RA) and soluble TNF-α receptor 1 (sTNFR1). Peripheral blood mononuclear cells (PBMC) were isolated for ex vivo generation of IL-2.[18,22] Sterile procedures were carried out on all samples to ensure no contaminants would interfere with the ELISA assays or ex vivo generation of IL-2. Plasma samples were centrifuged at 1,500*g* for 20 min at room temperature to remove cellular components before being stored in 1 ml aliquots at −20°C for ELISA assays or being admixed with trace metal free Ultrex II Nitric Acid (J.T. Baker Chemicals, Phillipsburg, NJ) and digested for Zn and copper analysis by flameless atomic absorption (AA).[18,22]

Laboratory Measurements

Aliquots of plasma samples were stored at −20°C until assayed for cytokines to ensure that both pre- and post-samples could be determined using the same high-sensitivity ELISA kits available from R&D Systems (Minneapolis, MN). Plasma Zn and Cu levels were determined by flameless AA spectrophotometry with a Zeeman background corrector (AA220Z Varian, Sugarland, TX).[18,22]

PBMCs were isolated by discontinuous density gradient using Histopaque 1077 (Sigma–Aldrich, St. Louis, MO)

FOI 26-3132 - Document 5

according to the manufacturer's protocol. Isolated PMNC were resuspended in incubation media consisting of RPMI-1640 supplemented with 10% fetal bovine serum, 1.5 gm/L sodium bicarbonate, 0.1% gentamicin, 2 mM L-glutamine and 50 μM β-mercaptoethanol. Cells were stimulated for 48 hr with 10 μg/ml PHA-p (Sigma, St. Louis, MO) at 37°C under an atmosphere of 85% air/5% CO_2. At the termination of the experiment, supernatants were harvested and assayed for IL-2 by ELISA (Quantikine ELISA, R&D Systems).

Characterization of Zn− Versus Zn+ Subjects

Zn adequate CF subjects (Zn+) were defined as those subjects with ≥90 μg/dl plasma Zn as determined by flameless AA at baseline. Subjects exhibiting inadequate Zn status (Zn−) had plasma Zn levels ≤89 μg/dl or two standard deviations below our normal database of 110 ± 10 μg Zn/dl.[18,22] Although all subjects exhibited plasma Zn levels within the clinically accepted "normal" range (70–120 μg/dl),[23] results from a number of our research studies have demonstrated that subjects exhibiting low Zn levels (<90 μg/dl) are more susceptible to infections and tend to have higher levels of plasma oxidative stress molecules and inflammatory cytokines even in the absence of outward infections.[18,22]

Statistical Analyses

Baseline data comparison between Zn adequate and Zn inadequate CF patients were determined by the Student's *t*-test. Data representing differences between pre- and post-values were analyzed using the Student's *t*-test for plasma cytokines Zn and Cu and ex vivo generation of IL-2, as well as for weight, height, hemoglobin, and FEV_1. Correlation between compliance and days of oral antibiotic usage was determined by regression analysis using Pearson r (GraphPad Instat Ver. 3.05 for Windows95/NT, San Diego, CA).

RESULTS

Overall, there were no statistical differences in the baseline parameters examined between Zn adequate and inadequate CF children with the exception of plasma Zn status (Table 1). Zn adequate CF patients also tended to have higher levels of the sIL-1RA, IL-1 β, and IL-6 and although the differences did not reach statistical significance (Table 1).

Zn supplementation administered to 12 CF patients (one subject dropped) resulted in a significant ($P = 0.05$) reduction in the number of days of oral antibiotics use per year compared to the 13 patients on placebo. However, the response to Zn supplementation was different for the Zn inadequate versus Zn adequate CF patients. Average days of antibiotic use for the Zn adequate CF subjects given Zn were not different from those given the placebo ($P = 0.55$; Table 2). This is in contrast to the response from the Zn inadequate CF subjects in which the effect of Zn supplementation was significantly greater ($P = 0.025$) compared to those given placebo (Table 2).

Compliance of Zn adequate CF subjects given Zn was less than placebo treated Zn adequate subjects (Table 2).

TABLE 1—Baseline Characteristics of Subject Population

Parameter	Zinc adequate subjects (n = 13)	Zinc inadequate subjects (n = 13)	*P*-value
Age (year)	12.0 ± 3.4 (7–17)[1]	11.8 ± 3.6 (8–18)	0.8854
Weight (kg)	37.58 ± 13.6 (23–67)	42.67 ± 13.0 (21–59.7)	0.3391
Height (cm)	146.53 ± 17.12 (124–178)	147.87 ± 14.44 (123–167)	0.8310
Hbg (g/dl)	13.66 ± 0.85 (12.2–15.2)	13.66 ± 0.68 (12.5–15.0)	0.9999
Pl.[2] Zn (μg/dl)	101 ± 9.8 (90–125)	82 ± 4.9 (73–89)	<0.0001
Pl. Cu (μg/dl)	106 ± 20 (80–150)	113 ± 20 (66–142)	0.3811
FVC-P[3]	83.85 ± 12.1 (65–98)	87.0 ± 10.8 (70–104)	0.4908
FEV_1P[4]	67.5 ± 17.7 (41–100)	76.77 ± 15.3 (54–98)	0.1660
FEF_{25-75} P[5]	48.69 ± 29.47 (10–109)	65.38 ± 32.43 (12–109)	0.1829
Gen. IL-2[6]	411 ± 529 (25–1590)	382 ± 695 (3–2370)	0.9058
Pl. sIL-1ra (pg/ml)	1501 ± 1880 (149–6900)	927 ± 938 (131–2995)	0.3384
Pl. sTNF-R1 (pg/ml)	1368 ± 887 (348–3017)	1179 ± 463 (716–2060)	0.5054
Pl. IL-1β (pg/ml)	2.48 ± 2.66 (0.35–7.1)	1.47 ± 1.08 (0.43–3.6)	0.2240
Pl. TNF-α (pg/ml)	2.84 ± 2.13 (1.5–9.3)	2.30 ± 1.05 (1.15–4.6)	0.4236
Pl. IL-6 (pg/ml)	6.18 ± 5.71 (1.8–18.6)	3.82 ± 4.99 (0.52–17.2)	0.2734
Pl. IL-8 (pg/ml)	440 ± 864 (0–2858)	581 ± 1427 (0–5552)	0.7639

[1]Values represent Mean ± SD (range).
[2]Pl. represents plasma values.
[3]FVC-P = forced vital capacity expressed as percent predicted.
[4]FEV_1P = forced expiratory volume in 1 sec, expressed as a percentage of predicted.
[5]FEF_{25-75} P = forced expiratory flow between 75 and 25 of FVC expressed as percent predicted.
[6]IL-2 generated ex vivo.

FOI 26-3132 - Document 5

TABLE 2— Number of Episodes Requiring Antibiotics, Number of Days of Oral Antibiotic Usage and Compliance in Zinc and Placebo Supplemented Subjects With Cystic Fibrosis

	Zinc adequate subjects			Zinc inadequate subjects		
Parameter	Placebo (n = 7)	Zinc (n = 5)	*P*-value	Placebo (n = 6)	Zinc (n = 7)	*P*-value
No. of episodes req. i.v. AB[1]	0.57 ± 0.53[2]	0.40 ± 0.55	0.559	0.17 ± 0.41	0.14 ± 0.37	0.915
No. of episodes req. oral AB[3]	3.28 ± 2.9	2.6 ± 0.89	0.574	3.16 ± 1.6	1.57 ± 0.97	0.049
%Compliance[4]	92.8 ± 7.6	66.7 ± 21.1	0.012	81.4 ± 18.8	70.3 ± 37.7	0.566
Avg. days/year Req. oral AB[5]	49.1 ± 36.6	37.4 ± 6.0	0.55	47.5 ± 24.3	20.5 ± 12.8	0.025

[1]Average number of episodes/year requiring intravenous (i.v.) antibiotics.
[2]Values represent Mean ± S.D.
[3]Average number of episodes/year requiring oral antibiotics.
[4]Average percent compliance.
[5]Average days/year requiring oral antibiotic use.

However, overall compliance of the two groups of Zn treated subjects was similar. Moreover, Pearson correlation coefficient indicated no correlation between compliance and number of days of oral antibiotic usage in all Zn supplemented subjects ($r^2 = 0.025$). There were no adverse events identified for any of the subjects in this study from oral or i.v. antibiotic use, Zn or placebo supplementation or any other parameter associated with this study.

Analysis of pre- versus post-values for each parameter resulted in non-significant percent change compared to baseline in selected parameters between the Zn supplemented and placebo supplemented group (Table 3). This was more likely due to the small number of patients and the large standard deviation within each group consisting of both Zn inadequate and and Zn adequate CF patients. In Zn treated CF patients, Zn supplementation was associated with less increase in plasma IL6 and IL-8, and a greater increase in plasma sIL-1ra and the ex vivo generation of IL-2 (Table 3). This may indicate an anti-inflammatory role of Zn in this group of patients.

TABLE 3— Percentage Increase (Decrease) in Selected Parameters Obtained From Zinc and Placebo Supplemented Cystic Fibrosis Subjects

Parameter	Placebo Tx (n = 13)	Zinc Tx (n = 12)	*P*-value[1]
Pl[2]. Zn (μg/dl)	8.6 ± 22[3]	6.1 ± 9.4	0.73
Pl. sIL-1ra (pg/ml)	1.9 ± 141	11.4 ± 101	0.84
Pl. sTNFR1 (pg/ml)	9.7 ± 56	8.1 ± 27	0.93
Pl. IL-1β (pg/ml)	86 ± 304	78 ± 188	0.94
Pl. TNF-α (pg/ml)	26 ± 93	32 ± 87	0.85
Pl. IL-6 (pg/ml)	173 ± 374	106 ± 192	0.57
Pl. IL-8 (pg/ml)	481 ± 1507	122 ± 337	0.41
Gen[4]. IL-2 (pg/ml)	55 ± 274	185 ± 275	0.24

[1]*P* values for difference between placebo and zinc supplemented group.
[2]Pl. represents plasma.
[3]Mean ± SD percent change from baseline.
[4]Ex vivo generation of IL-2 in PHA-p stimulated mononuclear cells.

DISCUSSION

A number of investigators have reported conflicting relationships between Zn status and malabsorption, impaired growth, or pulmonary function in CF patients.[5,19,21,24,25–28] Few studies found no correlation between Zn levels and nutritional or pulmonary status[19,25] Other reports found abnormal Zn status in certain patients with CF especially those with moderate to severe growth retardation and severe pulmonary disease.[26–28] Low plasma Zn in CF was also associated with low plasma IL-2 levels, and an impairment of NK cell activity and low thymulin activity.[21] Thymulin activity, necessary for the development of IL-2 producing T lymphocytes, was dependent upon the presence of the Zn molecule being present in the thymulin peptide structure.[29] An increase in the number of CD3+ lymphocytes positive for IL-2 after stimulation has been reported in CF but was not correlated with Zn status.[30] In this present study, isolated PMNC from Zn supplemented CF patients had increased ability of generating IL-2 over the time compared to those from subjects administered placebo.

While some studies showed an association between Zn deficiency, growth retardation and malnutrition in a subset of patients with CF, our group of CF subjects did not appear to be ''Zn deficient'' but had plasma Zn levels still within clinically acceptable normal range.[23] Based on our long term studies in trace metal research, we have found that subjects with plasma levels of <90 μg/dl are more likely to suffer adverse reactions to immunological challenges such as cold, flu and infections than those subjects with plasma Zn levels above this range. In this study, Zn inadequate CF subjects exhibited lower baseline levels of plasma sIL-1ra, sTNFR1, IL-1β, IL-6, and IL-8 than their Zn adequate counterpart. One hypothesis may be that in CF subjects with low plasma Zn levels, maintainence of immunological surveillance is decreased in the absence of acute exacerbation. In CF subjects with inadequate Zn status, Zn therapy was associated with

FOI 26-3132 - Document 5

significantly fewer days of oral antibiotics and insignificant change in plasma inflammatory cytokines pre- to post-therapy than their Zn adequate counterpart. However, the small number of subjects and large SD for selected parameters are not optimal for determination of a greater beneficial effect for Zn inadequate subjects compared to their Zn adequate counterpart. Changes in plasma Zn levels, weight, height, FEV1, and Hgb in CF receiving Zn were not different from the placebo treated CF group. This suggests that the dose of Zn (30 mg/day) may not have been sufficient to affect growth rate or other parameters of lung function due to possible suboptimal absorption of Zn from the gastrointestinal tract. Slightly higher dose of Zn (no greater than 45 mg) may produce a more favorable response on anthropometric, and lung function measures. In patients with SCD in which loss of Zn due to red cell destruction and hyperzincuria leaves approximately 70% of SCD subject with mild to moderate Zn deficiency, supplemental Zn in dosages of 50–75 mg/day are required to reduce incidence of infection and the presence of endogenous plasma inflammatory cytokines.[31] Supplemental Zn at 45 mg/day has been shown to reduce the incidence of infection and reduce plasma inflammatory cytokines in the elderly population even in the absence of active infection[18] and to reduce the presence of oxidative stress markers in young adults without adverse effects or changes in copper levels.[16] Various studies in developing countries that used relatively high doses of Zn, ranging from 88 to 150 mg per week, in pre-school children for up to 15 months have reported significant reduction respiratory and gastrointestinal infections with out adverse effects.[32,33]

Several studies reported increased plasma and bronchoalveolar lavage fluid (BALF) levels of various cytokines such as IL-8 in patients with CF even in newly diagnosed infants with and with out documented airway infections.[10,34–39] One report showed increased IL-8 in plasma and BALF and increased in sera IL-8 was associated with age in CF subjects as compared to serum IL-8 from normal children which decreases with age.[35] Along with IL-8, increased TNF-α is also found in BALF samples from CF subjects and similar to IL-8, is inversely correlated to lung function FEV1.[34] BALF levels of both IL-8 and TNF-α tend to decrease with antibiotic therapy. Compared to normal controls, other dysregulations of immune function have been reported in CF subjects such as decreased plasma IL-10 in response to antigenic stimuli,[40,41] increased plasma adhesion molecules sICAM and sE-selectin,[42] and decreased IL-8 production in isolated T cells.[30] In other studies, results reflect data obtained from CF populations separated into groups of responders versus non-responders based on the cytokine studied. However, rarely has the Zn status of these patients been considered even though some subjects with CF are known to exhibit lower plasma Zn levels. Studies performed in non-CF adults and elderly demonstrate that plasma levels of inflammatory cytokines and oxidative stress markers are reduced following Zn therapy[16–18,31] and provide support for Zn as an anti-inflammatory agent in CF.

Results of our research in humans and in cell models indicate the effect of Zn in reducing inflammatory cytokines is at the mRNA level and involves inhibition of NF-κB activation.[43] HL-60 cells, (a promyelocytic cell line) rendered Zn deficient and then stimulated with LPS, produce significantly greater amounts of TNF-α, IL-1β and IL-8 than those cells that were Zn sufficient.[43] Activation of the NF-κB pathway by *P. aeroginosa* in subjects with CF and in CF animal models can lead to an overproduction of adhesion molecules, inflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-8), and oxidative stress molecules, which in turn continue to provide stimulus for further NF-κB activation and the relentless vicious cycle.[44,45]

The most significant observation in this present study was the association of Zn supplementation with the reduction in the average days of oral antibiotic usage required to treat RTIs particularly in Zn inadequate subjects. It is possible that a more profound effect of Zn than inflammatory cytokine reduction was to modulate the airway environment. The effect of Zn on pulmonary function also include such events as inhibition of NADPH oxidase which generate extracellular superoxide anions[11] and the restoration of chloride secretion across the CF airway epithelia independent of changes in the CFTR as has been demonstrated in the CF mouse model.[13] In the CF mouse model, direct application of ATP and Zn was shown to trigger calcium entry via the P2X purinergic receptor channels leading to the activation of calcium-dependent chloride channels, circumventing the defective cyclic AMP dependent CFTR chloride channel.[13] Zn therapy can also modulate pulmonary sodium absorption via epithelial sodium channel (ENaC) directly and through the regulation of redox conditions.[46] The height of the airway surface liquid lining the apical side of the respiratory epithelium is tightly regulated and involves a balance between sodium absorption by ENaC and chloride secretion by the CFTR in normal children or by activation of the calcium-dependent chloride channel in CF with defective CFTR. Inducing ''near normal'' airway environmental conditions by Zn in CF would provide for suboptimal condition for infectious organisms.[47]

In summary, we have provided evidence that 30 mg/day supplemental Zn was associated with reduction in the number of days of oral antibiotics used to treat RTI and marginal reduction in the level of plasma inflammatory cytokines in children with CF. Our study has several limitations. The number of patients with CF was not large enough to enhance the potential differences between the treated groups. As a result, this study can be considered as

FOI 26-3132 - Document 5

a pilot study providing preliminary data for power analysis for future studies designed with larger number of subjects. Slightly higher doses of Zn therapy may be needed to statistically change anthropometric measures, lung function, the level of plasma inflammatory cytokines, and attenuate the severity of pulmonary exacerbations in subjects with CF. Finally, the effectiveness of Zn therapy may be optimally determined by assessing cytokine levels in BALF or sputum samples which would more directly representative of inflammation in the compartmentalized CF airways. Further investigations are needed to understand the role of Zn in mediating pulmonary infection in CF subjects.

ACKNOWLEDGMENTS

The authors are very thankful to CF Services Pharmacy for supplying Vitamax® tablets free of charge. We are also very grateful to Cathy Van Wagnen and her invaluable help as a research nurse during the course of the study period.

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FOI 26-3132 - Document 5

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Received: 29 May 2021 | Accepted: 31 May 2021

DOI: 10.1002/jmv.27122

FOI 26-3132 - Document 5

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Clinical study evaluating the efficacy of ivermectin in COVID-19 treatment: A randomized controlled study

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Abstract

Researchers around the world are working at record speed to find the best ways to treat and prevent coronavirus disease 2019 (COVID-19). This study aimed to evaluate the efficacy of ivermectin for the treatment of hospitalized mild to moderate COVID-19 infected patients. This was a randomized open-label controlled study that included 164 patients with COVID-19. Patients were randomized into two groups where Group 1 (Ivermectin group) included patients who received ivermectin 12 mg once daily for 3 days with standard care and Group 2 (control group) included patients who received standard protocol of treatment alone for 14 days. The main outcomes were mortality, the length of hospital stay, and the need for mechanical ventilation. All patients were followed up for 1 month. Overall, 82 individuals were randomized to receive ivermectin plus standard of care and 82 to receive standard of care alone. Patients in the ivermectin group had a shorter length of hospital stay (8.82 ± 4.94 days) than the control group (10.97 ± 5.28 days), but this was not statistically significant ($p = 0.085$). Three patients (3.7%) in each group required mechanical ventilation ($p = 1.00$). The death rate was three patients in the ivermectin group (3.7%) versus four patients (4.9%) in the control group without any significant difference between the two groups ($p = 1.00$). Although there was no statistically significant difference in any endpoints by ivermectin doses (12 mg/day for 3 days); there was an observed trend to reducing hospital stay in the ivermectin-treated group.

KEYWORDS

COVID-19, chloroquine, ivermectin, mortality, treatment

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by a novel coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was first recognized amid an epidemic of respiratory illness cases in Wuhan City, Hubei Province, China. It was originally reported to the World Health Organization (WHO) on December 31, 2019. The WHO declared COVID-19 a global pandemic on March 11, 2020.[1,2]

COVID-19 management depends on the severity of the disease. Mild cases usually recover at home, whereas moderate and

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FOI 26-3132 - Document 5

severe cases should be monitored closely and sometimes need hospitalization.[3–5]

Developing an effective therapy for COVID-19 infection is a complex process. Several immunotherapies, antiviral agents, and vaccines continue to be studied and developed as potential therapies for COVID-19.[4,5]

In March 2020, the WHO declared COVID-19 a global pandemic and an increasing number of cases of COVID-19 have been reported worldwide including in Egypt. The repurposing of commercially available COVID-19 pharmaceutical drugs has contributed to the production of hundreds of trials around the world.[1–11] Egyptian protocols to treat COVID-19 infection have mainly relied on many potential drugs, including, hydroxychloroquine, remdesivir, nitazoxanide, oseltamivir, favipiravir, lopinavir/ritonavir, and ivermectin.[11]

Ivermectin, in vitro, is an inhibitor of SARS-CoV-2 which is able to lead to approximately 5000-fold reduction in viral RNA at 48 h.[10] This beneficial mechanism of ivermectin on the COVID-19 virus was not known until recently and it was thought that it behaved in the same manner as other viruses. The nuclear importation of virus and host proteins has been known to be inhibited. Virus integrase protein and importin (IMP) alpha/β1 heterodimer is responsible for nuclear import, further increasing infection are also, inhibited.[10–26]

Ivermectin may be a safe, affordable, and readily available therapy of COVID-19. Therefore, the use of ivermectin warrants the rapid implementation of controlled clinical trials to assess the efficacy against SARS-CoV-2.[5] There were observational studies suggesting a beneficial effect of ivermectin in the treatment of COVID-19. So, we aimed in this study to evaluate the efficacy and safety of ivermectin in the treatment of COVID-19.

2 | METHODS

2.1 | Study design and participants

This was a 1:1 randomized, open-label, parallel-group clinical trial of ivermectin versus standard of care for the treatment of hospitalized patients with COVID-19. The study took place between March 2020 and October 2020 at Tanta and Assiut University Hospitals, two tertiary hospitals in Egypt. The study was conducted in isolation units in both hospitals.

All adult patients from ages 20 to 65 with mildly to moderately affected COVID-19 infection confirmed by pharyngeal swab polymerase chain reaction (PCR) were included in the study. Patients who had an allergy or contraindication to the drugs used in the study, pregnant and lactating mothers, and patients with cardiac problems were excluded from the study.

2.2 | Randomization

The included patients were randomized using a computer random number generator to select random permuted blocks with a block size of eight and an equal allocation ratio. Sequentially numbered, opaque, sealed envelopes were used to ensure concealment. Three members of the study team (Soliman S, Mai Khalaf, and Eslam Saber Esmail) recruited, enrolled, and assigned participants to a computer-generated randomization sequence, held by an independent observer. During randomization, the proportional allocation of each clinical stratum was equalized in both groups. The included patients were first stratified according to their age (18–25, 26–40, 40–55, and >55), then by their gender (inside each stratum into males and females) and then their associated morbidities (absent and present). We tried to keep the randomization equalized inside each stratum as much as we could.

2.3 | Procedures

Patients and physicians were aware of the assigned treatments. The patients were divided into two groups:

Group1: (Ivermectin group) 82 patients who received a single dose of oral ivermectin tablets (12 mg) every day for 3 days added to the standard protocol of treatment according to the Egyptian Ministry of Health (MOH) protocol of COVID-19 treatment.[11] Ivermectin tablets (Iverzine tablets, Unipharma) were used in the study.

Group 2: (Control group) 82 patients received standard protocol of treatment alone for 14 days. All patients were followed up for 1 month. All participants were submitted to the following demography: age, sex, weight, and height measurements and calculation of body mass index (BMI), history-associated symptoms and co-morbidities, clinical examination, and medication history. The following blood tests were done for all the patients in the trial including CBC analysis (WBCs-lymphocytes-PLTs-hemoglobin), liver function tests, Chest X-ray, PCR for pharyngeal swab and ECG, computed tomography according to CO-RADS score.[26]

The Egyptian MOH adopted a standard of care treatment protocol for patients with COVID-19. It included paracetamol, oxygen, fluids (according to the condition of the patient), empiric antibiotic, oseltamivir if needed (75 mg/12 h for 5 days), and invasive mechanical ventilation with hydrocortisone for severe cases if PaO_2 less than 60 mm Hg, O_2 saturation less than 90% despite oxygen or noninvasive ventilation, progressive hypercapnia, respiratory acidosis (pH < 7.3), and progressive or refractory septic shock.[11] Any side effects of the studied medication were assessed using by questionnaire done by the patients or their guardians.

2.4 | Outcomes

The primary outcome was the assessment of clinical efficacy of ivermectin in the form of the analysis of all-cause mortality within 1 month after randomization and the secondary outcomes were the length of hospital stay, the need for mechanical ventilation, and the assessment of the safety of ivermectin.

2.5 | Ethical considerations

The study was approved by the Tanta University faculty of medicine ethical committee and was registered on clinicaltrial.gov with registration number (NCT04403555). Informed consent was obtained from all participants in this study. Privacy of participants and confidentially of the data were assured. Risks and benefits were declared and any unexpected risks which appeared during the course of the research were cleared to participants and the ethical committee on time.

2.6 | Statistical analysis

The primary analysis was done based on an intention-to-treat basis including all randomly assigned individuals. By the time of planning this study, there were very few pre-print pilot studies of ivermectin on humans (e.g., The study by Gorial et al.[16]) The study included all the eligible patients who agreed to participate. The calculated post hoc sample power was 0.80 based the following inputs: two-tailed sample power, 0.44 effect size, 0.05 α error probability, and 82 as the sample size in each group (the outputs were: 3.02 as noncentrality parameter δ, 1.97 as critical t, 162 as df).[21]

The normality of the variables was tested by the Shapiro–Wilks test. Data were analyzed by Statistical Package for Social Sciences (SPSS) V. 23 and were expressed in number (No), percentage (%) mean ($\bar{x}$), and standard deviation (*SD*). Student's *t*-test was used for normally distributed continuous variables and Mann–Whitney's test for not normally distributed variables. A χ^2 test (with Z test to compare column proportions) was used to study the association between categorical variables, and whenever any of the expected cells were less than five, Fisher's Exact test was used. Binary logistic regression was used to ascertain the effect of the potential I risk factors on the patients' mortality. The regression model was a simple one including one variable at each time. The included risk factors were age, gender, smoking, alanine aminotransferase (ALT), albumin, creatinine, ferritin, C-reactive protein (CRP), need for mechanical ventilation, diabetes mellitus, and ivermectin treatment. All the variables were continuous except for gender, smoking, need for mechanical ventilation, presence of diabetes, and treatment with ivermectin (each was of two categories only). Two-sided p value of less than 0.05 was considered statistically significant.

3 | RESULTS

A total number of 170 patients with mildly to moderately affected COVID-19 infection confirmed by pharyngeal swab PCR were invited to the study. Eight patients refused to complete the study, 164 patients were enrolled and they were randomized to receive ivermectin plus standard of care (82 patients) and 82 to receive standard of care alone. All the patients continued the study medications to the end of the duration of treatment and follow-up.

TABLE 1 Patients' characteristics and clinical presentation at baseline

Variable	Ivermectin (n = 82) No. (%)	Control (n = 82) No. (%)	p value (of χ^2)
Age (mean ± *SD*)	42.38 ± 16.02	39.38 ± 16.92	0.23[a]
Gender			
Male	37 (45.1)	45 (54.9)	0.21
Female	45 (54.9)	37 (45.1)	
Diabetes	17 (20.7)	10 (12.2)	0.14
Hypertension	18 (21.9)	14 (17.1)	0.52
Comorbidities	36 (43.9)	45 (54.9)	0.16
Fever	52 (63.4)	43 (52.4)	0.16
Headache	34 (41.5)	21 (25.6)	0.42
Sore throat	22 (26.8)	26 (31.7)	0.49
Anorexia	19 (23.2)	23 (28.0)	0.47
Anosmia	12 (14.6)	9 (1.2)	0.48
Pallor	2 (2.4)	1 (1.2)	0.56[b]
Cyanosis	2 (2.4)	2 (2.4)	1.00[b]
Fatigue	55 (67.1)	46 (56.1)	0.14
Abdominal pain	22 (26.8)	16 (19.5)	0.27
Diarrhea	30 (36.6)	35 (42.7)	0.43
Vomiting	4 (4.9)	11 (13.4)	0.06
Cough	56 (68.3)	56 (68.3)	1.00
Dyspnea	20 (24.4)	26 (31.7)	0.29
CT according to CO-RADS score[26] (level of suspicion for pulmonary involvement of COVID-19)			0.67
0 (Not interpretable)	28 (34.1)	23 (28.0)	
1 (Very low)	12 (14.6)	18 (22.0)	
2 (Low)	31 (37.8)	27 (32.9)	
3 (Equivocal)	9 (11.0)	10 (12.2)	
4 (High)	2 (2.4)	3 (3.7)	
5 (Very high)	0 (0.0)	1 (1.2)	

Abbreviations: COVID-19, coronavirus disease 2019; CT, computed tomography; *SD*, standard deviation; χ^2, chi squared test.

[a]Mann–Whitney test.

[b]Fisher's Exact test.

The studied groups were matched for age and gender. The mean was 42.38 ± 16.02 year for Group 1 and 39.38 ± 16.92 for Group 2 (p = 0.231). Males represented 45.1% of Group 1 and 54.9% of Group 2 (p = 0.212) (Table 1). They had no significant difference regarding the presence of comorbidities (p = 0.160). Among Group 1, 20.7% had diabetes and 21.9% had hypertension or other cardiovascular diseases, while among Group 2, 12.2% had diabetes and 17.1% had

FOI 26-3132 - Document 5

TABLE 2 Clinical course of the disease

Clinical course	Ivermectin (n = 82) No. (%)	Control (n = 82) No. (%)	p value
Length of hospital stay (in days) mean (*SD*)	8.82 ± 4.94	10.97 ± 5.28	0.08[a]
Need for mechanical ventilation	3 (3.7)	3 (3.7)	1.00[b]
Fate within 1 month			
Survived	79 (96.3)	78 (95.1)	1.00[b]
Died	3 (3.7)	4 (4.9)	

[a]Mann–Whitney test.
[b]Fisher's Exact test.

hypertension or other cardiovascular diseases. The clinical signs and symptoms of the disease did not show any significant difference between the two groups. Moreover, the laboratory tests did not show any significant difference between the two groups.

The ivermectin group had a shorter hospital stay (8.82 ± 4.94 days) than the control group (10.97 ± 5.28) but this was not statistically significant (p = 0.085). Three patients (3.7%) needed mechanical ventilation in each group (p = 1.00). Death among patients of the two groups did not show any significant difference either, as three patients (3.7%) died in the ivermectin group and four patients (4.9%) died in the control group (p = 1.00) (Table 2).

The univariable regression analysis showed that the patients' age, ALT, creatinine, ferritin, and CRP were independently related to the patients' mortality (p = 0.004, 0.001, < 0.001, 0.008, and < 0.001, respectively) (Table 3). Mild side effects were recorded; they were minimal in the ivermectin group with nausea in one patient and diarrhea in two patients. Ivermectin was not significantly related to the patients' mortality (p = 0.700).

TABLE 3 Univariable binary logistic regression analysis of predictors of mortality (each raw represents a separate model with one predictor)

	Univariate			
			95% CI	
Variable	p value	OR	Lower	Upper
Age	0.004	1.100	1.032	1.174
Gender	0.583	0.652	0.141	3.008
Smoking	0.998	–	–	–
ALT	0.001	1.031	1.013	1.049
Albumin	0.217	0.332	0.053	1.944
Creatinine	<0.001	226.346	11.483	4457.78
Ferritin	0.008	1.004	1.001	1.006
CRP	<0.001	1.022	1.010	1.034
Need for mechanical ventilation	0.99	–	–	–
DM	0.07	4.156	0.874	19.75
Ivermectin	0.70	0.741	0.160	3.417

Abbreviations: ALT, alanine transaminase; DM, diabetes mellitus; CI, confidence interval; OR, odds ratio.

4 | DISCUSSION

The purpose of this randomized controlled trial was to determine the effectiveness and safety of ivermectin in COVID-19 therapy. Our study involved 164 patients who met the enrollment criteria and were enrolled and randomized to the ivermectin group (n = 82) or the control group (n = 82). The baseline demographic and clinical presentations in the two studied groups were similar without any significant difference. The laboratory parameters and the radiological findings were not significantly different either. In this study, the two groups did not show any statistically significant difference in clinical outcomes. Although there was no statistically significant difference, a trend to reducing the length of hospitalization was observed in the ivermectin. This favorable outcome of ivermectin was in accordance with Ahmed et al.[12] and Elgazzar et al.,[13] who observed that early incorporation of ivermectin to the standard of care was very successful for the treatment of COVID-19 patients with shortened hospital stay duration; and was even associated with higher viral clearance.[12] Also, a pilot study by Chaccoura et al.[22] showed a tendency to lower viral loads in the ivermectin group as well as a tendency to lower IgG titers. This result may be due to its effect on the suppression of a number of inflammatory cytokines contributing to the "storm of cytokines" and then all disease squalls.[10]

In a nonrandomized study by Gorial et al.,[16] the addition of ivermectin to hydroxychloroquine significantly shortened the length of hospital stay in comparison to hydroxychloroquine alone. Also, three recent clinical trials confirmed the beneficial impact of the addition of ivermectin to the standard of care for the outcome of decreasing hospital stay.[17] However, Podder et al.[18] and Chachar et al.[19] did not demonstrate any benefit of the addition of ivermectin to the treatment of COVID-19 in relation to our observations.

The ivermectin group did not show any significant difference either in the need for mechanical ventilation (p = 1.00) or the patients' mortality (p = 1.00) from the control group. In contrast, Elgazzar et al.,[13] showed that ivermectin treatment was associated

FOI 26-3132 - Document 5

with lower mortality during treatment of COVID-19, especially in patients with severe pulmonary involvement.

The addition of ivermectin to the treatment protocol in our study did not show any significant association with the patients' mortality by univariate binary logistic regression analysis ($p = 0.700$, odds ratio 0.471; 95% confidence interval: 0.160–3.417). However, other risk factors like the patient's age, serum ALT, creatinine, ferritin, and CRP being significantly related to all-cause mortality may be attributable to the small dose of ivermectin. Accessible pharmacokinetic evidence from clinically significant and excessive dosage trials indicates that the concentration of ivermectin needed to suppress SARS-CoV-2 in humans with known dosing regimens is unlikely to be reached in serum and tissue.[20–30] Pott-Junior et al.[24] as well as Babalola et al.[25] supported our results that higher doses of ivermectin may have a greater antiviral effect, but also showed that supra-lethal toxic doses are unnecessary to inhibit viral replication.

Owing to the scarce resources of our developing country, the limitations of research outcomes may depend primarily on the patients' health conditions, the need for intensive care unit entry, and the consequences of mortality, and not the viraemic response. Another drawback is the small ivermectin dosage which we recommend to be increased in further trials. Small sample size, lack of blindness, and lack of placebo group were additional limitations to our study.

In conclusion; the usage of ivermectin did not achieve significance for any of the endpoints. However; there was an observed trend to reducing hospital stay in the ivermectin-treated group. These findings may suggest using ivermectin as an add-on therapy to protocols used for the treatment of COVID-19. However, these results are needed to be validated in a larger prospective follow-up study.

CONFLICT OF INTERESTS

All the authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Sherief Abd-Elsalam was responsible for the concept, design, definition of intellectual content, and manuscript preparation. Sherief Abd-Elsalam, Rasha Abdel Noor, and Rehab Badawi were responsible for literature search, manuscript review, and manuscript editing. Sherief Abd-Elsalam, Rasha Abdel Noor, Rehab Badawi, Mai Khalaf, Eslam Saber Esmail, Mohamed Samir Abd El Ghafar, Mohamed Elbahnasawy, Ehab F. Moustafa, Sahar M. Hassany, Mohamed Alboraie, Haidi Karam-Allah Ramadan, Mohamed Alboraie, Mohamed Alboraie, Ahmed Cordie, and Gamal Esmat were responsible for data acquisition, Clinical studies. Shaimaa Soliman and Sherief Abd-Elsalam were responsible for data analysis, statistical analysis. All authors have been read and approved the final version of the manuscript.

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FOI 26-3132 - Document 5

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How to cite this article: Abd-Elsalam S, Noor RA, Badawi R, et al. Clinical study evaluating the efficacy of ivermectin in COVID-19 treatment: A randomized controlled study. *J Med Virol*. 2021;93:5833-5838. https://doi.org/10.1002/jmv.27122

FOI 26-3132 - Document 5

Journal of Antimicrobial Chemotherapy (2006) **58**, 256–265
doi:10.1093/jac/dkl224
Advance Access publication 1 July 2006

JAC

Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines

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The pharmacokinetics of tetracyclines and glycylcyclines are described in three groups. Group 1, the oldest group, represented by tetracycline, oxytetracycline, chlortetracycline, demeclocycline, lymecycline, methacycline and rolitetracycline is characterized by poor absorption after food. Group 2, represented by doxycycline and minocycline, is more reliably absorbed orally, while group 3, represented by the glycylcycline tigecycline, is injectable only, with an improved antibacterial spectrum compared with the tetracyclines. Though incompletely understood, the pharmacodynamic properties of the tetracyclines and glycylcyclines are summarized.

Keywords: pharmacokinetics, pharmacodynamics, tetracyclines

Introduction

The tetracyclines including the glycylcyclines represent a large group of antibacterials, some of which were first introduced into clinical practice in the 1950s (tetracycline) while others have recently been approved (the glycylcycline, tigecycline) or are in pre-clinical development (the aminomethylcycline, PTK 0796). Given the duration over which these drugs have been developed and clinically used it will be no surprise that the available information on the pharmacokinetics of individual agents is patchy and comparisons are often difficult. There is also a lack of robust information on the pharmacodynamic properties of the class; the last member to be introduced into clinical practice, minocycline, in the early 1970s, pre-dates the development of modern pharmacodynamics concepts by more than a decade.

In general the tetracyclines can be divided into three groups based on their pharmacokinetic and antibacterial properties.

Group 1: This group consists of the older agents which have reduced absorption and are less lipophilic than newer drugs in group 2. Examples are tetracycline, oxytetracycline, chlortetracycline, demeclocycline (demethyl chlorotetracycline), lymecycline, methacycline and rolitetracycline. All can be administered orally except for rolitetracycline.

Group 2: These drugs are more or less completely absorbed and are 3–5 times more lipophilic than drugs in group 1. This may improve their tissue distribution but convincing data is absent. They are available in oral and intravenous formulations. Examples are doxycycline and minocycline.

Group 3: This group includes the developmental compounds aminomethylcyclines (e.g. BAY73-6944/PTK 0796) which are yet to enter clinical trials and the recently approved glycylcycline tigecycline. These antibiotics are active *in vitro* against many bacteria with acquired resistance to tetracyclines.

In this review we will concentrate on the pharmacokinetics of each group in turn and then discuss the pharmacodynamics of the class as a whole. The focus is primarily on the older tetracyclines as there are a number of reviews of tigecycline already published and more importantly new data will become available for this drug over the next few years. The data presented on tetracyclines should make it possible to place tigecycline in an appropriate context. With the older agents, it is important to note that the antibiotic assay methodologies used may not meet present standards; this may be partly responsible for the difference noted between studies.

Pharmacokinetics

Group 1: tetracycline, oxytetracycline, chlortetracycline, demeclocycline, lymecycline, methacycline and rolitetracycline

HPLC assays and spectrofluorometric methods are available for most of these agents[1,2] but many investigators have used microbiological assays.[3,4] A range of formulations is available, but rolitetracycline is the only one to be available as intravenous only. In many European markets these drugs are only available

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Table 1. Pharmacokinetics of tetracyclines[6–13]

Agent	Formulations	Percentage absorption	Doses (mg)	Peak concentration (C_{max}) (mg/L)	Time to peak concentration (t_{max}) (h)	Half-life ($t_{1/2}$) (h)	Protein binding (%)	Volume of distribution (L/kg or L)	Elimination (%)	
									urinary	faecal
Tetracycline	po/iv	77–88	250	2	2–4	6–11	55–64	1.3 L/kg or 108 L	30	20–60
			300	2.5	3	7.8				
			500	3–5	2	8.5				
Oxytetracycline	po/iv	58	250	2	3	9.2	27–35	128 L	–	50
			500	4						
Chlortetracycline	po	25–30	500	1.4	3	5.6	50–55	100 L	–	>50
Demeclocycline	po	66	150	1.2	4	–	75–91	1.7 L/kg or 121 L	40	43
			300	1.7	4	10–17				
			500	2.5	6	13				
Lymecycline	po	–	300	2.1	3	8	–	–	25	–
Methacycline	po	58	300	2–3	3	14	75–78	–	33	–
Rolitetracycline	iv	none	275	2–4	–	5–8	–	–	>50	–
			300	4–6						

in oral formulations. Topical preparations are also marketed, but are not discussed here.

Absorption, distribution, metabolism and excretion

Absorption is variable ranging from 0% to almost 90%; however, for most agents it is in the range 25–60%.[5] Serum concentrations rise slowly after oral administration with absorption occurring in the stomach, duodenum and small intestine. C_{max} (mg/L) depends on dose, but is generally in the range 1–5 mg/L (Table 1). t_{max} is in the range 2–4 h except for demeclocycline whose C_{max} is delayed until 4–6 h.

All these tetracyclines form insoluble complexes with calcium, magnesium, iron and aluminium, which markedly reduces absorption.[14] The effect of disease on the absorption of these drugs is unknown. Protein, fat and carbohydrate meals reduce the absorption of tetracycline by about 50%.[2] The volume of distribution (V) for these agents is in the order of 1.3–1.7 L/kg or a total volume of distribution of 100–130 L. These data imply some concentration in tissues; however, most data on tissue penetration are of poor quality, making firm conclusions about their relative distribution difficult. Protein binding is variable. None of these agents undergoes metabolism with the exception of tetracycline, 5% of which is excreted as the metabolite Δ-epitetracycline.[1] Unchanged drugs are excreted by renal and bilary routes. Renal elimination (CL_R) is related to glomerular filtration for most agents, the exception being chlortetracycline.[15,16] The amount of drug excreted in the urine is <50%; rolitetracycline is said to have high renal elimination. Greater than 40% appears in the faeces after biliary elimination and most drugs have some enterohepatic circulation.[5] Biliary concentrations can exceed blood by a factor of 5.[17]

Serum concentration time profiles

Tetracycline concentrations in blood follow a plateau-shaped course with a slow rise and a slower drop,[6] the serum half-life ($t_{1/2}$) being in the range 6–10 h for tetracycline, oxytetracycline, chlortetracycline and lymecycline. The half-lives of demeclocycline and methacycline are longer, 10–17 h. There has been little formal assessment of dose-linearity with these drugs, but serum concentrations clearly increase with increasing dose.[12] There is very little data on areas under the serum time curves, but 300 mg of tetracycline or lymecycline produced AUCs of 26.9 ± 6.0 and 21.9 ± 4.3 mg·h/L, respectively.[11] Five hundred milligrams of tetracycline on an empty stomach, as tablets or dissolved in water, produced average AUC in the range 55–75 mg·h/L.[2]

Special groups

There are no data on the effect of age, sex, obesity or low body weight on the pharmacokinetics of these drugs. There are no data on the effect of infection, other co-morbidities or liver impairment with the agents. Exercise increases serum concentrations and reduces renal clearance of tetracycline but the clinical significance of this is hard to judge.[18]

Renal failure is known to reduce the elimination of tetracycline[16] but not chlortetracycline where the serum half-life in patients with chronic renal failure is similar to those with normal renal function.

Group 2: doxycycline and minocycline

There is a significant published database on the pharmacokinetics of doxycycline[19–22] and minocycline[20]—therefore, these agents will be discussed separately.

Doxycycline

HPLC, biological and fluorometric assays for doxycycline have been described.[2,20,23,24] At present, only the oral formulation is available in most countries, though an iv form of doxycycline has been developed and used clinically.

Absorption, distribution, metabolism and excretion

Doxycycline is said to be almost completely absorbed with a bioavailability of more than 80%[19] with an average of ~95%.[20] However, some authors comparing intravenous (iv) and oral (po) doses of doxycycline felt absorption was lower—in the range 73–77%.[25] Absorption takes place in the duodenum.[20]

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Table 2. Pharmacokinetics of doxycycline

Dose (mg) and route	Single dose (SD) or multi-dose (MD)	Peak concentration (C_{max}) (mg/L)	Time to peak concentration (t_{max}) (h)	Half-life ($t_{1/2}$) (h)	Area under serum time curve (AUC) (mg·h/L)	Volume of distribution (V) (L or L/kg)	Clearance (mL/min)			Reference
							total	renal	non-renal	
100 po	MD	2.0 ± 1.0	1.8 ± 1.1	12 ± 3	13 ± 5	–	–	–	–	26
100 po	SD	1.7	2.5–3	14.3 ± 14.6	37–40	–	–	–	–	27
200 po	SD	5.9 ± 1.8	2.6 ± 0.8	16 ± 4	113 ± 46	–	–	–	–	28
200 po	SD	5.2 ± 1.5	2.7 ± 0.8	13 ± 5	90 ± 16	0.7 ± 0.1	–	–	–	29
200 po	SD	4.8 ± 0.3	–	–	81 ± 5	–	–	–	–	18
200 po	SD	5.7 ± 2.6	2.6 ± 1.0	25 ± 19	123 ± 81	–	30 ± 13	–	–	30
200 po	SD	2.6 ± 0.2	2.9 ± 0.4	20 ± 6	41 ± 6	62 ± 18	–	–	–	31
200 po	SD	2.6 ± 0.7	–	14 ± 7	54 ± 32	–	78 ± 38	–	–	32
200 iv	SD	–	–	16.3	92 ± 18	50 ± 9	37 ± 6	23 ± 4	14 ± 4	33
200 iv	SD	9.3	–	14	112	–	–	–	–	34
200 iv	MD	1.9–3.5	–	17 ± 7	61 ± 23	79 ± 11	63 ± 20	–	–	35
100 iv	6.9 ± 0.7	–	16 ± 2	8.8 ± 9	0.7 ± 0.1	30 ± 3	14 ± 3	14 ± 2	–	36

The half-life of absorption is 0.85 ± 0.41 h.[29] The peak concentration (C_{max}, mg/L) varies with dose being 15.3 mg/L 4 h after a dose of 500 mg orally (Table 2).[37]

Doxycycline–metal ion complexes are unstable at acid pH, therefore more doxycycline enters the duodenum for absorption compared with the earlier compounds.[38] In addition, food has less effect on absorption than on absorption of earlier drugs[10] with doxycycline serum concentrations being reduced by ∼20% by test meals compared with 50% for tetracycline.[2] The volume of distribution is 50–80 L or 0.7 L/kg (Table 2) and the protein binding is 82–93%,[19] but may be lower in some patient groups.[28] There is little good quality data on tissue penetration. In healthy volunteers, the area under the serum time curve (AUC) ratio of AUC blister/AUC serum is 54% in a suction blister fluid model.[26] All other tissue penetration depends on single time point estimations; however, penetration into sputum is 8–28% estimated over 16 h.[39] Penetration into saliva is poor while biliary concentration exceeds serum by many fold.[17,22] Penetration as assessed by a 3 h post-dose concentration measurement after a single peri-operative dose of doxycycline in orthopaedics indicated levels below that of serum in bone, skin, fat, tendons and muscle.[40] Concentrations are highest in the liver, kidney and digestive tract, that is the excretory organs.[20] There is no significant metabolism and no metabolites have been found in man.[19,20] However, in an interaction study with rifampicin, some patients had reduced doxycycline C_{max} and AUC, suggesting there may be some hepatic metabolism.[32] Doxycycline is eliminated unchanged by both the renal and biliary routes. Bile concentrations may be 10–25 times those in serum.[41] About 35–60% is excreted in urine and the remainder in faeces[12,18,30,34] (Table 2).

Serum concentration time profiles

Doxycycline is slowly absorbed orally, taking 2–3 h to reach peak concentrations. The elimination half-life is long, ranging from 12 to 25 h depending on the study (Table 2). Average AUCs for 200 mg/day oral doses range from 40–123 mg·h/L and 61–112 mg·h/L for iv doses. There is little data on dose-linearity or the effects of multiple doses on the pharmacokinetics of doxycycline.

Special groups

The pharmacokinetics of doxycycline have been studied in the elderly, undernourished patients with hyperlipidaemia, infected patients and patients with renal impairment. There are no data on the impact of sex, pregnancy, lactation or liver impairment on doxycycline pharmacokinetics.[20] The impact of age on the pharmacokinetics of 200 mg iv doxycycline was studied in 25 patients over 65 years old—mean age 76, range 65–90 years. The $t_{1/2}$ was 15 ± 9 h, AUC (mg·h/L) 194 mg·h/L and the volume of distribution 25 ± 9 L. Serum concentrations were higher than those reported in other groups with a C_{max} of 8–30 mg/L and concentrations at 10 h in the range 5–10 mg/L. The reason for these increased serum concentrations and reduced volumes of distribution is unclear.[42] Undernourished patients dosed with 200 mg iv doxycycline had lower AUC, shorter serum half-lives, serum concentrations and protein binding. Total and non-renal clearances were increased.[36] Compared with control patients, those with hyperlipidaemia had increased serum AUC and C_{max}.[31] The clinical importance and underlying pathophysiology are not determined. Exercise in young, healthy volunteers had the effect of increasing the C_{max} of 200 mg doxycycline from 4.8 ± 0.3 to 6.5 ± 0.6 mg/L and the AUC from 81 ± 5 to 106 ± 3 mg·h/L.[18] The reasons for this are unclear.

Several studies have been conducted in patients with various degrees of renal impairment and those with end stage renal disease.[28,34,41,43]

Patients with chronic renal failure have reduced urinary recovery of doxycycline and increased faecal recovery.[34,41,43] However, the serum half-life is not greatly increased, being reported in the range 20–25 h. As expected, AUCs are mostly increased for a 200 mg dose, being on average in the range 120–196 mg·h/L.[34] The C_{max} is unchanged[34] but protein binding is significantly reduced in patients with end stage renal disease.[28]

There is very limited information on the effects of haemodialysis, ∼10% of the drug was removed in a 7 h dialysis session.[43]

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Dose modification is probably not needed in renal impairment as the hepatic and non-hepatic gastro-intestinal elimination compensate for loss of renal clearance.

Klastersky *et al*.[44] studied the serum of doxycycline 200 mg iv, multiple doses, in patients with severe hospital infections. Peak concentrations were in the range 5–6 mg/L and troughs 1–2 mg/L, not unlike healthy volunteer data.

In more recent times, the pharmacokinetics of doxycycline in 17 patients recovering from severe *Plasmodium falciparum* malaria have been reported.[45] Patients received 200 mg of the drug orally and blood samples were drawn in the acute and convalescent phases. In the acute phase, the C_{max} (mg/L) was 3.2 (1.6–7.7); $AUC_{0–24}$ (mg·h/L) 32 (18.7–79.7); $t_{1/2}$ (h) 10.5 (6.9–17.9); and volume of distribution (L/kg) 1.4 (0.6–3.0). Equivalent values in the convalescent phase were as follows: C_{max} 4.4 (1.5–8.6) mg/L; $AUC_{0–24}$ 48.6 (18.3–69.8) mg·h/L; $t_{1/2}$ 11.6 (8.5–17.2) h and volume of distribution 0.9 (0.5–2.7) L/kg.

Minocycline

HPLC and biological assays are used to measure minocycline.[3,46] At present, there are only oral formulations available clinically as capsules, tablets, modified release capsules and suspension. An iv formulation has also been investigated.[46]

Absorption, distribution, metabolism and excretion

Minocycline is almost completely absorbed (95–100%),[20] mainly in the stomach, duodenum and jejunum. Unlike other tetracyclines, food does not appear to have an effect on either the C_{max} or AUC.[47] The kinetics of absorption of minocycline follow the same broad pattern as for tetracycline, that is after oral administration a rise to C_{max} by 2 h.[48] The C_{max} increases, with increasing dose being 0.65 mg/L after 50 mg, 2.2 mg/L after 150 mg, and 3–3.6 mg/L after a 200 mg dose[12,47–49] (Table 3). After intravenous administration, the C_{max} concentrations are 3–8.75 mg/L after a 200 mg dose.[29,51–53] Absorption is reduced by iron and antacids containing calcium and magnesium.[46] There is little data on the volume of distribution of minocycline, values of 80–115 L have been reported[53] or 1.17 L/kg.[50] Protein binding is 76%[51] and the effect of disease on protein binding has not been studied. As with other tetracyclines, there is very little good quality data on tissue penetration. However, a tissue/serum concentration ratio of >10 has been reported for liver and bile; 5–10 for duodenum, gall bladder and thyroid; and <2 for colon, bladder, prostate, uterus, breast, skin, lymph nodes and veins.[51] No minocycline has been detected in saliva and concentrations of <50% serum in CSF have been reported.[52] Minocycline is unusual for a tetracycline in that it has a variety of metabolites; up to six have been described, some of which have antibacterial activity and are found in urine. The principal metabolite is 9-hydroxyminocycline; the other two main metabolites are mono-N-demethylated derivatives. Epimerization of minocycline also results in the formation of 4-epiminocycline.[54] Little minocycline is recovered in the urine—probably 5–12% of the dose. Faecal elimination accounts for about 20–35% of the dose.[12,48,51,53] Renal clearance is about 2.2–1.2 mL/min.[50]

Serum concentration time profiles

Minocycline reaches C_{max} after 2–3 h post-oral dose and then has a prolonged serum half-life of 12–18 h.[34,48,49,53] A summary of the pharmacokinetic parameters is shown in Table 3. The AUC_{24} after a 200 mg oral dose is ∼45 mg·h/L and the equivalent $AUC_{0–\infty}$ is about 70 mg·h/L.[47,48] After a 200 mg iv dose, the $AUC_{0–\infty}$ were 70–86 mg·h/L.[50] There is little known on dose-linearity with minocycline or the effect of multiple dosing on pharmacokinetic parameters. Interesting is in one study which reported the data where the serum half-life was increased after multiple doses.[50] The reason for this is not known but may be related to saturation of excretory pathways.

Special groups

There are no data on the effects of age, sex, changes in body mass, co-morbidity or infection on the pharmacokinetics of minocycline except for a small number of studies carried out in patients with renal impairment and end stage renal disease. Renal impairment and end stage renal disease have little effect on the serum concentrations and serum half-life or AUC of minocycline[34,50,53,55] in both single dose and short multi-dosing studies. As may be expected, the urinary recovery in renal failure is <1% total dose.[34]

Group 3: Aminomethylcyclines

BAY 73-6944/PTK 0796 is a compound being investigated and is currently in pre-clinical development. In animal models it is substantially unbound in plasma at concentrations ranging from 0.5 to 10 mg/L. In the presence of liver homogenates it appears to be metabolically stable with no detectable loss of compound over 60 min.[56,57]

Table 3. Pharmacokinetics of minocycline

Dose and route	Single doe (SD) or multi-dose (MD)	Peak concentration (C_{max}) (mg/L)	Time to peak concentration (t_{max}) (h)	Half-life ($t_{1/2}$) (h)	Area under serum time curve (AUC) (mg·h/L)	Reference
50 mg po	SD	0.65	–	–	2.1 (0–6 h)	47
200 mg po	SD	3.1	2	17	43.9 (0–24 h)	48
200 mg po	SD tablet	3.5	2–4	12.9	47.6 (0–24 h)	49
	SD capsule	3.6	2–4	13.1	46.7 (0–24 h)	
200 mg iv	SD	3.0	–	17	85.8 (0–8 h)	50
200 mg iv	MD	4.1	–	21	69.8 (0–8 h)	34
200 mg iv	SD	–	–	14.6	67 (0–7 h)	

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Table 4. Mean pharmacokinetic parameters of tigecycline after various single iv doses [mean (% CV)]

	1 h infusion						
	12.5	25	50	75	100	200	300
C_{max} (mg/L)	0.11 (10)	0.25 (25)	0.38 (17)	0.57 (14)	0.91 (29)	1.64 (18)	28 (17)
AUC (mg·h/L)	0.75 (68)	2.26 (45)	2.56 (21)	3.67 (27)	6.40 (10)	12.42 (23)	17.86 (10)
CL (L/h/kg)	0.29 (27)	0.20 (50)	0.28 (14)	0.29 (16)	0.20 (13)	0.25 (22)	0.25 (11)
V_{SS} (L/kg)	2.8 (34)	6.4 (20)	6.4 (31)	7.5 (10)	8.6 (18)	11 (25)	12 (20)
$t_{1/2}$ (h)	11 (84)	32 (64)	18 (21)	21 (25)	38 (14)	42 (28)	46 (13)
CL_R (L/h)	–	–	–	–	2.6 (26)	3.0 (50)	2.7 (23)

C_{max}, peak concentration; AUC, area under the serum time curve; CL, clearance; V_{SS}, volume of distribution at steady state; $t_{1/2}$, half-life; CL_R, renal clearance.

Table 5. Mean pharmacokinetic parameters of tigecycline after various iv doses—multi-dosing [mean (%CV)]

	Tigecycline dose (mg)					
	25		50		100	
	day 1	day 10	day 1	day 10	day 1	day 10
C_{max} (mg/L)	0.26 (14)	0.32 (17)	0.49 (17)	0.62 (15)	0.82 (15)	1.17 (15)
AUC (mg·h/L)	0.80 (8)	1.48 (18)	1.44 (14)	3.07 (12)	2.39 (13)	4.98 (19)
$t_{1/2}$ (h)	–	49.3 (72)	–	36.9 (32)	–	66.5 (34)
CL (L/h/kg)	–	0.20 (17)	–	0.20 (9)	–	0.24 (20)
V_{SS} (L/kg)	–	8.6 (23)	–	7.2 (7)	–	9.1 (32)

C_{max}, peak concentration; AUC, area under the serum time curve; $t_{1/2}$, half-life; CL, clearance; V_{SS}, volume of distribution at steady state.

Glycylcyclines (tigecycline)

Tigecycline has been assayed by HPLC but more recently by a liquid chromatography/mass spectrometry (LC/MS) assay.[58,59] Pharmacokinetic data are available from healthy volunteer studies and also from Phase 3 clinical trials of therapy in skin and soft tissue infection and intra-abdominal sepsis, hence the data available are much more comprehensive than for the earlier drugs. As yet, there is only an intravenous formulation of tigecycline which is infused over a 1 h period.

Absorption, distribution, metabolism and elimination

There are no data on absorption of tigecycline, but its oral bioavailability is reported to be limited. A light breakfast did not have a significant effect on iv tigecycline pharmacokinetics.[60] The volume of distribution is large with initial values of volume of distribution at steady state (V_{ss}) being >10 L/kg.[61] Total volumes of distribution are ~350 L in women and 500 L in men.[58]

The more recent presentation of a dose ranging study of 12.5–300 mg as single doses or multi-dosing has added significantly to this earlier report.[62,63] For single dose exposures of 12.5, 25, 50, 75, 100, 200 and 300 mg the V_{ss} (L/kg) were 2.8, 6.4, 6.5, 7.5, 6.8, 13 and 12, respectively, indicating some dose dependency. In the multi-dose studies the V_{ss} (L/kg) for the 25, 500 and 100 mg doses were 8.6, 7.2 and 9.1, respectively (Tables 4 and 5). The reasons for these differences are at present unclear; however, in the dose range used in man 100 mg loading, then 50 mg 12 hourly, the volume of distribution is somewhere in the range 2.5–7.0 L/kg.[63] Given tigecycline's large volume of distribution there must be tissues into which it is concentrated. As yet tissue penetration data are limited; however, in 10 healthy volunteers using a cantharidin skin blister model and measuring the AUC_{0-12} in both blister fluid and serum after a 100 mg iv dose, the penetration was 74% (AUC_{0-12} blister fluid = 1.6 ± 0.2 mg·h/L; AUC_{0-24} serum = 2.2 ± 0.3 mg·h/L). The equivalent C_{max} concentrations were 0.17 mg/L in blister fluid and 0.82 mg/L in serum.[59]

Further studies in adult humans who received 100 mg iv tigecycline have indicated concentrations in lung tissue of 0.8 mg/L at 4 h post-dose, 0.2 mg/L at 8 h, 0.4 mg/L at 12 h and 0.4 mg/L at 24 h. There was significance between subject variability in measured drug concentrations. CSF concentrations in non-inflammed meninges were low being 5% 1 h post-infusion and 41% after 24 h. As may be expected, bile concentrations were high, ranging from 0.16–4.37 mg/L, with bile/serum ratios in the range 606–1997. This is consistent with biliary excretion of tigecycline.[64]

Radiolabelled tigecycline has been administered to Sprague-Dawley rats as a single infusion to study the drug distribution.[65] As expected peak radioactivity was seen at the end of infusion in all the tissues. AUC values were highest in the bone, bone marrow, salivary gland, thyroid, spleen and kidney. The tissue AUC/plasma AUC was >1 for most tissues over the 168 h of the experiments. The tissue/plasma ratios were 2046 for bone, 149 for bone marrow, 90 for thyroid, 45 for spleen and 24 for liver. The high ratio in bone may be related to calcium binding.[65]

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Tigecycline protein binding ranges from 73% at 0.1 mg/L to 79% at 1 mg/L, the reason for this atypical pattern is as yet unknown.[63] The metabolism of tigecycline in man has been studied using radiolabelled tigecycline.[66] There are two major metabolic pathways, the main route being glucuronidation and its inactive epimer producing 5–20% of the radioactivity in human serum. An N-acetyl-9-aminominocycline metabolite is also formed to a less degree.[66] At least five human metabolites have been described in urine, serum, faeces and plasma.[60] Less than 15% of tigecycline is excreted unchanged in the urine[61,67] and renal clearance accounts for <20% total clearance.[60] Radiolabelled tigecycline studies in man indicated that 59% of the radioactivity was found in faeces and 32% in urine. This presumably means that, like some other tetracyclines, there is biliary excretion and perhaps also an enterohepatic circulation.

Serum concentration pharmacokinetic profile

The serum values of the C_{max} (mg/L), AUC (mg·h/L), serum half-life (h) and clearance (L/kg/h) for single doses of 12.5, 25, 50, 75, 100, 200 and 300 mg and 25, 50 and 100 mg multiple doses are shown in Tables 4 and 5. The proposed dosing in man is a 100 mg loading dose followed by 50 mg 12 hourly. This will give a C_{max} of 0.62 ± 0.09 mg/L and an AUC at steady state of 3.1 ± 0.4 mg/L·h. The C_{max} of the 100 mg dose is 0.9 mg/L and the 50 mg in multi-dosing 0.62 mg/L. The half-life of the 100 mg dose is 22 h in the single dose study and 66 h in the multi-dose, equivalent values for the 50 mg dose are 18 and 38 h, indicating lengthening of the half-life with multiple doses. Drug clearance is usually ∼0.2 L/h/kg irrespective of the dose or number of doses. There is rough linearity with dose in terms of AUC and C_{max} values. Steady state is reached within 3 days.[62]

Using the single and multiple dose data from Phase 1 subject populations, pharmacokinetic models have been developed.[68] For single dose data a three-compartment model with zero order input and first order elimination adequately described the data, despite the presence of secondary peaks in some profiles. However a two-compartment model provided the most unbiased estimates of $AUC_{0–12}$ and is therefore most appropriate to multi-dose studies in patients.

Special groups

The effects of age, gender, excretory organ failure and infection on the pharmacokinetics of tigecycline have been studied. The effect of age and gender has been studied in healthy volunteers in three age groups, 18–50, 65–75 and >75 years old.[58] A single 100 mg 1 hourly infusion was given and the mean C_{max} was 0.85–1.0 mg/L in all groups. The lowest AUC was in males under 50 years (mean AUC 4.2 mg·h/L) and the highest in those over 75 years (means AUC 5.8 mg·h/L). The mean AUC for women was ∼5 mg·h/L in all the groups. Studies have been conducted on subjects with severe renal impairment (creatinine clearance < 30 mL/h) and those with end stage renal disease maintained by haemodialysis. These data were compared with those from healthy volunteers. A single 100 mg infusion over 1 h was administered. The C_{max} and AUC values are shown in Table 6. Haemodialysis had no effect on the pharmacokinetics of tigecycline and only 5% of unchanged drug was recovered from the urine of those with severe renal impairment compared with 16% from the healthy volunteers.[67]

Table 6. Pharmacokinetics of tigecycline 100 mg infusion in healthy subjects, those with severe renal impairment and those with end stage renal disease

	Peak concentration (C_{max}) (mg/L)	Area under the serum time curve (AUC) (mg·h/L)
Healthy volunteers	0.60	3.3
Creatinine clearance < 30 mL/min	0.6	4.7
End stage renal disease on haemodialysis	0.96	4.0

There are no published data as yet on the impact of liver impairment on the pharmacokinetics of tigecycline. A non-compartmental pharmacokinetic analysis was performed on 15 patients in the Phase 3 study in complicated skin and soft structure infections.[69] The dosing regimen was 100 mg loading dose on day 1 followed by 50 mg 12 hourly until day 14.

The patients' weights varied from 57 to 200 kg; mean 80 kg. The C_{max} was 0.63 ± 0.28 mg/L, C_{min} 0.16 ± 0.05 mg/L, AUC_{ss} (0–12 h) 3.04 ± 0.81 mg·h/L, total clearance 17.5 ± 4.2 L/h and CL/kg 0.22 ± 0.06 L/h/kg. These data are in very close agreement with data from treated patients with intra-abdominal sepsis and from healthy volunteers in Phase 1 and Phase 2 studies.[63,70]

Co-administration of digoxin plus tigecycline or warfarin plus tigecycline in healthy male volunteers had no impact on the pharmacokinetics of either agent.[71,72]

Pharmacodynamics

Pharmacodynamics is the relationship between measurements of antimicrobial exposure in body fluids with the antibacterial and toxicological effects of the drug.[73] The most important measure of drug potency is still the minimum inhibitory concentration (MIC, mg/L); however, several other antibacterial effect measures are important. These include the pattern of bacterial killing, persistent effects and data from *in vitro* and animal pharmacokinetic/pharmacodynamic models.[74,75] Pharmacodynamic indices (AUC/MIC, C_{max}/MIC, t > MIC) can be related to bacteriological outcomes and specific pharmacodynamic index sizes used to predict clinical outcomes and establish clinical breakpoints.[76]

In general the pharmacodynamics of tetracyclines is understudied in comparison with some drug classes such as β-lactams, fluoroquinolones and aminoglycosides. The pattern of the antibacterial killing effect of doxycycline, minocycline and tigecycline has been studied using time–kill curve methods. Doxycycline produces a 0.5 log reduction in viable count of *Staphylococcus aureus* over 24 h at a concentration of 2× MIC. Increasing the concentration to 16× MIC results in 1–2 log kill. Against *Streptococcus pneumoniae* doxycycline produces no killing over 24 h at 2× MIC but this increases to >4 log kill at the 16× MIC concentration. Doxycycline produces no net effect against *Escherichia coli* at 4× MIC concentration over 24 h, but a 3 log kill at MIC 8–16.[77] The patterns of killing of minocycline and tigecycline were compared against *S. aureus*

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(n = 9), *E. coli* (n = 6) and *Klebsiella pneumoniae* (n = 6) in time–kill curves at 4× MIC concentration over 24 h. Minocycline produced a –1.5 ± 1.0 log kill against *S. aureus*, –2.0 ± 0.8 log kill against *E. coli* and +0.3 ± 1.8 log growth with *K. pneumoniae*. Equivalent values for tigecycline were –2.0 ± 1.3 log kill for *S. aureus*, –0.7 ± 0.7 log kill for *E. coli* and +0.4 ± 1.5 log growth for *K. pneumoniae*.[92]

At the concentration designed to mimic those achievable in man, tigecycline 2 mg/L, 0.5–0.7 log reductions in *Enterococcus faecium* and 0.7–1.4 log reductions in glycopeptide intermediate *S. aureus* were observed over 24 h.[78]

MBCs (mg/L) are 2-fold higher than the MIC for *S. pneumoniae* and 2–4 times the MIC for *S. aureus* of both minocycline and tigecycline. In contrast with *Enterococcus faecalis* tigecycline's MBC is >16 times the MIC.[79,80] Tigecycline produces ≤0.5 log killing against *E. faecalis* with most strains in time–kill curves over 24 h;[81] however, kill is enhanced by the addition of serum.[77]

Bacterial inoculum appears to have a modest effect on tigecycline MICs which are 1–2 dilutions higher with 10- to 100-fold increases in inoculum.[82] Data for other tetracyclines are absent.

Persistent antibiotic effects have been studied with tetracyclines *in vitro* and *in vivo*; however, the significance of persistent antibiotic effects such as the post-antibiotic effect (PAE) remains to be established. Doxycycline has a PAE against *S. aureus*, *S. pneumoniae* and *E. coli* which increases with increasing concentrations of exposure. At 2× MIC to 4× MIC concentrations the PAE for *S. aureus* is 0.7–1.2 h, *S. pneumoniae* 2.2–2.8 h and *E. coli* 1.0–1.4 h.[77] Tigecycline has been reported to have a longer PAE than minocycline against *S. aureus* and *E. coli* by an average 0.6–2 h at 8× MIC concentrations.[83]

In vivo using a murine model, tigecycline at a dose of 3 mg/kg single dose, C_{max} 0.42 mg/L, AUC 0.68 mg·h/L, and $t_{1/2}$ 0.25 h had PAE of 8.9 h against *S. pneumoniae* and 4.9 h against *E. coli*.[79] Tigecycline's PAE against *E. faecalis* is 1–4.5 h at 1–20 × MIC concentrations using time–kill *in vitro* studies.[81]

There is very little data on the use of *in vitro* pharmacokinetic or animal pharmacokinetic/pharmacodynamic models to describe the antibacterial effects of tetracyclines. Using a hollow fibre *in vitro* model to simulate free drug serum concentrations of oral minocycline 200 mg/day in man, the 24 h antibacterial effect was a –1.8 ± 0.2 log reduction in count by 24 h against UK EMRSA-16.[84]

Similar data to those of Bowker *et al*.[84] were reported by Garrison and Neumiller[85] in their *in vitro* model where tigecycline at concentrations associated with human dosing produced a 1.5 log drop in viable counts at 24 h. Tigecycline produced a greater antibacterial effect against fluoroquinolone-resistant *S. pneumoniae* than levofloxacin and against vancomycin-resistant enterococci than vancomycin.[84]

Van Ogtrop *et al*.[79] studied minocycline and tigecycline in a murine neutropenic thigh infection model against a number of strains of *S. pneumoniae*, *S. aureus* and *E. coli*; tigecycline's MICs were 0.06–0.12, 0.12–0.5 and 0.12 mg/L, respectively. The dose of tigecycline for a 24 h static effect was 0.8–5.9 mg/kg/day with *S. pneumoniae* but 25 times greater for *S. aureus* and *E. coli* (4.3–23 mg/kg/day) despite similar MIC values. Against *K. pneumoniae* the bacteriostatic dose was higher yet at 65–151 mg/kg/day. These data with that from time–kill curves suggest that the drug exposures to clear different bacterial species with tigecycline may be different, with *S. pneumoniae* requiring the lowest exposures, *S. aureus* and *E. coli* higher and *K. pneumoniae* higher yet. In this murine model AUC and $t >$ MIC could be related to antibacterial effect using non-linear regression analysis. The drug exposure to produce 80% of maximal effect was calculated as a free drug time greater than MIC of more than 50% of the dosing interval for tigecycline.[79]

Table 7. Successes and failures in man with iv doxycycline according to bacterial species and expected MIC_{50}

Species	MIC_{50} (mg/L) of representative strains	Success	Failure
S. pneumoniae	0.1	16	1
Group A streptococci	0.2	2	0
S. aureus	0.3	4	0
E. coli	12.0	3	2
Klebsiella/Enterobacter spp.	25.0	5	7
Proteus spp.	>50.0	2	9

More recently, based on likely drug exposures in man, tetracyclines have been classified as AUC driven agents.[73] As yet no AUC/MIC static effect targets have been published for any tetracyclines or tigecycline; such data from animal or *in vitro* models would be of help in determining clinical breakpoints.[76]

There are four studies relating tetracycline serum concentrations, pathogen MIC and species to clinical outcomes. Klastersky *et al*.[44] used iv doxycycline to treat a group of about 50 patients with a variety of infections including urinary tract infection, pneumonia, wound infection and septicaemia. Peak serum doxycycline concentrations were in the range 5–6 mg/L and trough concentration was 1–2 mg/L. Table 7 shows the successes and failures by species and the MIC_{90}s of representatives of those species performed by the authors. Of those bacteria which had an MIC of ≤0.3 mg/L (26) 25 infections were cured while if the MIC was ≥12 mg/L (30) 12 infections were cured.

In a different indication, Q fever endocarditis, 16 patients were studied over 1 year. *Coxiella burnetii* MIC, doxycycline serum concentrations and serological response (phase 1, *C. burnetii*) antigens were correlated. Those patients with a serum doxycycline to MIC ratio of ≥1 showed a more rapid decline in phase 1 antibodies than those with values between 0.5 and 1. Serum AUC values were not calculated and it was obviously difficult to generalize from this infection to others.[86]

The relationship between tigecycline AUC, MIC and microbiological outcome has been explored using data from three trials in complicated skin and soft tissue infection.[87,88] Two different dose regimens were used: 100 mg load plus 50 mg 12 hourly and 50 mg load plus 25 mg 12 hourly. The serum $AUC_{0–24}$ at steady state was median 5.16, range 2.81–9.36 mg/L (high dose) and median 2.33, range 1.49–4.98 (low dose). The pathogens isolated were divided into several groups, the most useful for the analysis being all Gram-positive pathogens (n = 36). One patient who failed tigecycline had a mesh graft for a hernia repair which was not removed. This patient was, very reasonably, excluded from outcome analysis. If the AUC/MIC value was <25, 5 patients failed therapy and 10 were cured, if the AUC/MIC was >25, no patients failed and 20 were cured. Classification and regression tree (CART) analysis identified an AUC/MIC of 12.3 for both

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microbiological and clinical responses. In a further analysis including patients with polymicrobial infection and MICs of tigecycline up to 16 mg/L the probability of cure was >0.9 if the AUC/MIC was >30.[87,88]

Given an AUC/MIC breakpoint of ~12–18 for infection mainly caused by *S. aureus*, the pharmacodynamic breakpoint would be ≤0.25 mg/L,[89] which is in close agreement with the breakpoint suggested from animal models of *S. pneumoniae*, *S. aureus* and *E. coli* infection.

A similar analysis was performed on patients in three trials of complicated intra-abdominal infection (n = 71). Patients received 100 mg iv tigecycline and then 50 mg twice daily, and the median AUC was 5.6 mg·h/L. APACHE score predicted microbiological and clinical outcomes and an AUC/MIC of 6.96 was significant in CART analysis for both microbiological and clinical outcomes. An AUC/MIC of >30 was associated with >90% chance of success in the final model.[90] These data were used to try to develop a pharmacodynamic breakpoint for *E. coli*. Several AUC/MIC targets were used, 6.96, 8.4 and 11.1; there was a 95% probability that the true AUC/MIC target was between 5.7 and 11.5. For an MIC of 0.5 mg/L, the target attainment rates for AUC/MIC ratios of 6.96, 8.4 and 11.1 were 94%, 83% and 54%, respectively.[91]

Conclusions

Tetracycline and glycylcycline pharmacokinetics and pharmacodynamics represent a relatively under-investigated but interesting area of antimicrobial chemotherapy. Until recently our understanding was poor and little progress had been made until tigecycline entered clinical development. We are still unclear as to the relative importance of serum versus tissue levels in predicting outcomes for the tetracyclines and glycylcyclines but this may be important given their large volumes of distribution. Much work could be done to improve our pharmacokinetic/pharmacodynamic knowledge of these agents, and as tigecycline enters clinical use it is to be hoped that this experience will add to our knowledge in the area.

Transparency declarations

APM receives lecture fees from Wyeth Pharmaceuticals.

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Journal of Bangladesh College of Physicians and Surgeons
Vol. 38, COVID-19 (Supplement Issue), July 2020

A Case Series of 100 COVID-19 Positive Patients Treated with Combination of Ivermectin and Doxycycline

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Abstract:

Background: A definitive treatment of SARS CoV-2 is yet to arrive and the human death toll rises exponentially globally. In this health emergency, it might be useful to look into the old therapies which could be effective against the virus. In vitro research showed Ivermectin could decrease the concentration of coronavirus 4000 to 5000 folds in living lung tissue.

Aim: In this prospective study a combination of Ivermectin and Doxycycline will be evaluated therapeutically to treat COVID-19 patients.

Methods: 100 COVID-19 patients were enrolled in this study with a predefined inclusion and exclusion criteria. RT- PCR of the SERS-CoV-2 will be done at designated government hospitals. The clinical features and response to treatment were noted according to a dedicated protocol.

Results: In this study male and female were 64 and 36 respectively, the age ranged between 8 to 84 years. Retesting was done between 4 and 18 days of starting medication. All patients tested negative and their symptoms improved within 72 hours. There were no noticeable side effects.

Conclusion: Combination of Ivermectin and doxycycline was found to be very effective in viral clearance in mild and moderately sick COVID-19 patients. Medical societies and institutions should undertake larger multi center studies to validate and recommend this combination therapy to include in national guidelines.

Keywords: Covid-19, Doxycycline, Ivermectin

Bangladesh Coll Phys Surg 2020; 38: 10-15)
DOI: https://doi.org/10.3329/jbcps.v38i0.47512

Background:

One hundred years after the Spanish flu pandemic in 1918-20, the whole world is again facing devastation due to the Covid-19 (SARS CoV-2) pandemic. This virulent virus SARS CoV-2 has high infectivity, morbidity and remarkable fatality rate. No specific treatment or vaccine has been invented to save mankind yet. Scientists, physicians and concerned multidisciplinary professional including political leaders are united to explore a quick effective treatment and vaccine before it is too late. Empirical applications of some drugs have been assumed to work with success, but without having a clinical trial, they cannot be validated. Until anything comes up, it might be useful to repurpose old therapies which could be effective against the virus.

In the beginning of April 2020, Caly et al. from Monash University, Australia published their research article stating that a single dose of Ivermectin (an anti-parasitic drug) could decrease the concentration of coronavirus in vitro. In the lab, Caly et al. bathed Vero-hSLAM cells with Ivermectin at a concentration of 5μM from 2 hours post-infection SARS-CoV-2 isolate Australia/VIC01/2020 until the conclusion of the experiment. SARS-CoV-2 RNA was determined by RT-PCR at days 0-3 in both supernatant and cell pellet experiments. The authors noted 93-99.8% reduction in viral RNA for Ivermection versus DMSO control at 24h in the supernatant (released virions) and cell associated viral RNA (total virus) respectively. They also describe by 48 hours about 5000 fold reduction of viral RNA and maintenance of effect at 72 hours.[1]

They concluded that the human dose needed to be ascertained in further studies. In the current SARS-CoV-2 pandemic, this news was a ray of hope.

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FOI 26-3132 - Document 5

Methodology:

Study design

Observational /Cross-sectional study

Study Population

All RT-PCR SARS- CoV-2 positive patients fulfilling the selection criteria. Mild and moderate cases purposively selected from Bangladesh Medical College.

Selection criteria

Inclusion Criteria

- Subjects within age group 5 years and above
- With either sex, male or female
- Confirmed cases of Covid-19 by RT-PCR test
- Patients who are classified as asymptomatic,
- Mild and moderate cases with typical symptoms
- Patients who are not already treated with any other antiviral drugs

Exclusion criteria

- Patients who are severe and critically ill.
- Patients with chronic liver diseases
- Patients with pregnancy and on lactation
- Children less than 5 years of age or less than 15kg weight.

Procedure

100 patients RT-PCR confirmed cases of SARS CoV-2 met the selection criteria and was enrolled in the study. They were given a combination treatment of Tab Ivermectin and Cap Doxycycline along with supportive treatment. The dose of ivermectin was 0.2 mg/kg single dose. Doxycycline 100 mg daily was given to patients aged 8 years and above for 10 days. Patients were given the choice to go to a COVID 19 designated hospital but they went home and continued treatment. Follow up was done every day for all symptomatic patients over telephone about remission of symptoms. RT-PCR test was repeated with sample of nasal swab for all patients according to availability of testing centers between 4 to 18 days. Six weeks after testing negative, we plan to follow up on the patient about their health conditions.

Result:

This observational study, consisting of 64 males and 36 females was conducted from April to May 2020 (Figure 2) in Bangladesh Medical College. The oldest patient was 84 years and the youngest one was 8 years with most patients between the ages of 21 to 40 years (Figure 3). Patients were divided in 3 groups: Mild (73), Moderate (20) and Severe (7), based on their symptoms. From the severe patients, three had fever more than 103 Fahrenheit for seven days with severe cough and lung infiltrates , three had severe loose motion and one had uncontrolled diabetes. Out of the rest, 20 patients had moderate symptoms of mild fever (100 Fahrenheit) and mild cough. Moreover, 73 had mild symptoms of malaise, sore throat, loss of smell, loss of taste, and body ache. Fifty percent symptomatic improvement of mild to moderate patients was seen between 3rd to 5th day after starting treatment. All 7 severe patients' symptoms subsided by 50 percent by 7th day of treatment. Retesting was done between 4 to 18 days of starting medication (Figure 4). Twenty five patients underwent retesting between 4th to 8th days, 51 between 9th to 13th days and 24 between 14th to 18th days from starting medication. All of the patients tested negative. None needed intensive care admission and no deaths were reported.

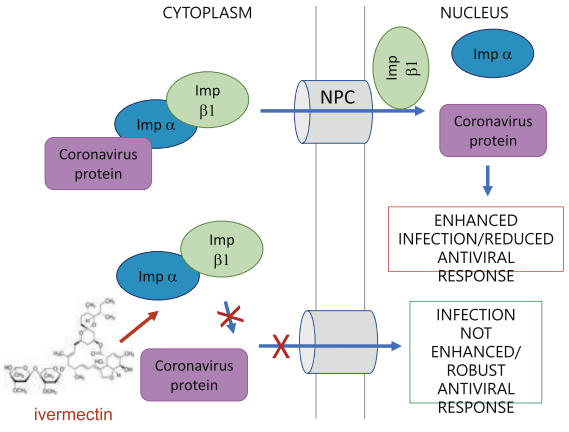


Fig 1: *Schematic of Ivermectin's proposed antiviral action on coronavirus .*

FOI 26-3132 - Document 5

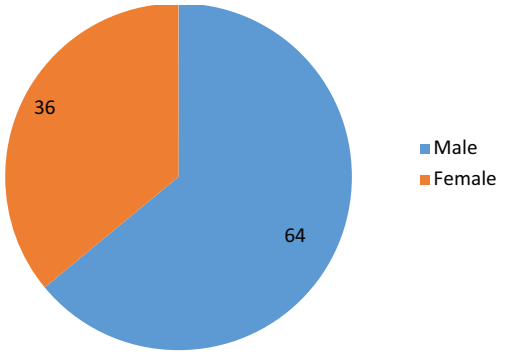


Fig.-2: *Gender of Covid-19 Positive patients (N=100)*

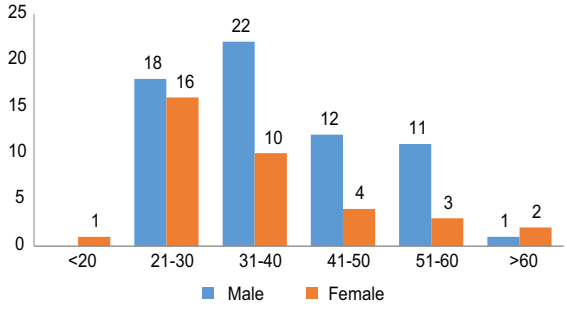


Fig.- 3: *Male and female ratio in different age groups of Covid-19 positive patients (N=100)*

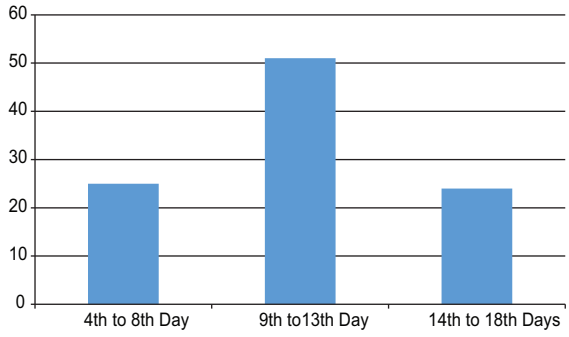


Fig.-4: *Number of Patients testing Negative on Days after Starting Treatment (N=100)*

Discussion:

An elderly female colleague with co-morbidities, who tested positive for SARS-CoV2, was referred to the Medicine Out Patient Department. As she was asymptomatic, she was advised isolation but she wanted to be treated without Hydroxychloroquine and Azythromycin fearing cardiac toxicity. She was informed about the recent findings on Ivermectin and agreed to take an oral combination of 200mcg/kg single dose of Ivermectin along with Doxycycline 100mg orally daily for 10 days. She tested negative in seven days.

This observation correlates with the findings of Caly et al.[1] We had given the standard single dose of Ivermectin that is normally given for the treatment of scabies and head lice although a very high dose was used in vitro. Doxycycline, a trusted antibiotic, anti-malarial, anti-typhus, and anti-viral which also decreases cytokine production without any cardiac toxicity has been a good combination in our small observational study[2]. We only treated asymptomatic and mostly mildly symptomatic patients.

Ivermectin was discovered in 1975 and came into medical use in 1981.[3,4] It is on the World Health Organization's List of Essential Medicines.[5] Ivermectin is a FDA approved drug, it is used for prevention, treatment, and control of river blindness (onchocerciasis) in populations where the disease is common. It is also used for treatment of Strongyloidosis, enterobiasis, Trichuris trichura, Loa Loa, Scabies, human lice, malaria and is also known to have wide-spectrum antiviral activity against number of viruses under in-vitro conditions. [6,7,8,9] SARS-CoV-2 is a single stranded RNA virus which is closely related to SARS coronavirus (SARS-CoV). Recent study on Ivermectin against SARS-CoV-2 under in-vitro conditions revealed that it can inhibit the viral replication. The single treatment of this drug was able to reduce the virus up to 5000-fold in Vero-hSLAM cells bathed with Ivermectin within 48 hours. However, no further reduction was reported with further increase in time period i.e up to 72 hours. Moreover, no toxicity was seen with the drug at any point of time[10]. Mechanism by which Ivermectin responded against the SARS CoV-2 virus is not known and was believed to be working similarly as it acted on other viruses. It was known to inhibit the nuclear import of viral and host proteins. Integrase protein of viruses and the importin IMPα/β1 heterodimer was responsible for IN nuclear import which further increases the infection. As most of the RNA viruses are dependent

FOI 26-3132 - Document 5

upon IMPα/β1 during infection, Ivermectin acts on it and inhibits the import with the increase in antiviral response [1,10].

Fig 1 shows a schematic of Ivermectin's proposed antiviral action on coronavirus. IMPα/β1 binds to the coronavirus cargo protein in the cytoplasm (top) and translocates it through the nuclear pore complex (NPC) into the nucleus where the complex falls apart and the viral cargo can reduce the host cell's antiviral response, leading to enhanced infection. Ivermectin binds to and destabilizes the IMPα/β1 heterodimer thereby preventing IMPα/β1 from binding to the viral protein (bottom) and preventing it from entering the nucleus. This, likely, results in reduced inhibition of the antiviral responses, leading to a normal, more efficient antiviral response[1].

Ivermectin is well tolerated by uninfected humans. It is primarily metabolized in the liver by CYP450-3A47 and has a plasma half-life of 16 hours and is almost exclusively excreted in faeces with minimal clearance by the kidneys. Therefore, it does not require dose adjustment for people with renal failure. There have been some reports of a mild anticoagulation effect. However, this is usually not significant enough to alter coagulation parameters such as the prothrombin ratio.

Side effects of oral Ivermectin are rare and usually minor. These include transient tachycardia, flushing, nausea and lightheadedness. More severe neurological side effects are theoretically possible in rare susceptible individuals. Moreover, FDA describes some of the side-effects that may be associated with Ivermectin include skin rash, vomiting, diarrhea, stomach pain, facial or limb swelling, neurologic adverse events (dizziness, seizures, confusion), sudden drop in blood pressure, severe skin rash potentially requiring hospitalization and liver injury (hepatitis) [11]. The safety of Ivermectin in pregnant women has not been studied and such use is not recommended. Studies in animals have shown an increase in birth defects. Safety and effectiveness in children below 5 years and under 15 kg and pregnant women have not been established. Ivermectin passes into breast milk and use during breast feeding is not recommended[12]. No significant drug interactions with Ivermectin are recognized.

In vitro studies showed Doxycycline to exert anti inflammatory effects at low (20 - 40mg/day) and high (100 or 200mg/day) doses with inhibitory action on metalloproteases and modulating effects of pro inflammatory cytokines IL 6, IL 8 and tumor necrosis factor alpha. The anti inflammatory properties of Doxycycline and other components of tetracycline has been demonstrated for several inflammatory airway diseases, including, acute respiratory distress syndrome. Thereby, low Doxycycline doses have been shown to be more effective than high doses to prevent induction of pro inflammatory cytokines (such as IL 6) in inflammatory diseases[13]. Doxycycline is rapidly and almost completely absorbed after oral administration and has half life of 16-18 hours. Based on the available evidence, we believe Tetracyclines may be effective agents in the treatment of Covid-19 due to their ability to chelate Zinc compounds on matrix metalloprotienases (MMP) on which coronaviruses rely heavily for survival, cell infiltration, cell to cell adhesion and replication, many of which has Zinc as part of their MMP complex.[14] It is a safe and inexpensive drug with a minimal toxicity[14]. Doxycycline is largely excreted unchanged both in the bile and urine and the dose does not require adjustment in patients with renal failure, because of their enterohepatic circulation this drug may remain in the body for a long time after cessation of therapy. Doxycycline can produce GI irritation most commonly after oral administration. Tolerability can be improved by administering it with food. Doxycycline can be safely used in children aged 8 years and above[15]. Currently global scenario is not stable as there is no effective drugs could be prescribed under sufficient evidence based clinical trials. Already efficacy and effectiveness of Hydroxychloroquine is questionable[16,17,18.] As Doxycycline is safe to use in treatment of ARDS it alone could be a better option for Covid-19 treatment.

FOI 26-3132 - Document 5

Conclusion:

It is too early in this pandemic to claim major successes of the effectivity of our combination therapy (Ivermectin and Doxycycline) as our number is small and there is no control group. Nevertheless, the results encourage us to continue the clinical study for people of the world in this improbable crisis as each and every life counts.

We urge the different medical societies and International organizations to take up this unique observation and quickly give it a trial and clearance because these drugs are FDA approved for many years with excellent safety and efficacy reviews. At the very least, using them in the first few days of being positive with or without symptoms may save the admissions of those 20% who suffer and the unnecessary deaths on ventilators. We can also stop the community transmission by asymptomatic carriers by treating the asymptomatic COVID-19 positive patients. Above all it is a very cheap combination, and if fast tracked by a quick trial would cost pennies and save a lot of lives, specifically designed for resource-poor settings.

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FOI 26-3132 - Document 5

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Cureus

Open Access Original Article

FOI 26-3132 - Document 5

DOI: 10.7759/cureus.9658

Clinical Outcomes of Early Treatment With Doxycycline for 89 High-Risk COVID-19 Patients in Long-Term Care Facilities in New York

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Abstract

Rationale

Due to the cluster and associated comorbidities in residents of long-term care facilities (LTCFs), COVID-19-associated morbidity and mortality are significantly increased. Multiple therapeutic options, including hydroxychloroquine (HCQ) and azithromycin (AZI), were tried initially to treat moderate to severe COVID-19 and high-risk patients in LTCFs, but they were abandoned due to unfavorable reports. As a less toxic option, we initiated treatment with doxycycline (DOXY) very early in the course of illness. DOXY has antiviral, cardioprotective, immunomodulatory, and anti-inflammatory properties, but the efficacy of early intervention with DOXY in high-risk COVID-19 patients in LTCFs is unknown.

Objective

The goal of this retrospective study is to describe the clinical outcomes of high-risk COVID-19 patients with moderate to severe symptoms in LTCFs after early intervention with DOXY.

Design

Case-series analysis

Setting

LTCFs in New York

Participants

This observational study examines 89 patients who were diagnosed with COVID-19 from March 18 to May 13, 2020.

Exposure

All patients who were diagnosed with COVID-19 received DOXY and regular standard of care

Received 07/15/2020
Review began 07/25/2020
Review ended 07/26/2020
Published 08/11/2020

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How to cite this article
Alam M M, Mahmud S, Rahman M M, et al. (August 11, 2020) Clinical Outcomes of Early Treatment With Doxycycline for 89 High-Risk COVID-19 Patients in Long-Term Care Facilities in New York. Cureus 12(8): e9658. DOI 10.7759/cureus.9658

within 12 hours of the onset of symptoms. Additionally, four patients received meropenem, three patients received Zosyn, two patients received linezolid, and two patients received Bactrim DS. Four patients were on chronic ventilator support. No patients received any steroids or any other antiviral or immunomodulatory agents. The majority of the patients received zinc and calcium supplements as well.

Main outcomes and measures

Assessed measures were patients' characteristics, fever, shortness of breath (SOB), cough, oxygen saturation/pulse oximetry (POX), radiologic improvements, laboratory tests, DOXY side effects, hospital transfers, and death.

Results

Eighty-nine (89) high-risk patients, who developed a sudden onset of fever, cough, SOB, and hypoxia and were diagnosed with COVID-19, were treated with DOXY (100 mg PO or intravenous (IV) for seven days) and regular standard of care. Eighty-five percent (85%) of patients (n=76) demonstrated clinical recovery that is defined as resolution of fever (average 3.7 days, Coeff = -0.96, p = 0.0001), resolution of SOB (average 4.2 days), and improvement of POX: average 84% before treatment and average 95% after treatment (84.7 ± 7% vs. 95 ± 2.6%, p = 0.0001). Higher pre- and post-treatment POX is associated with lower mortality (oxygen saturation (Spo2) vs. Death, Coeff = -0.01, p = 0.023; post-Spo2 vs. Death, Coeff = -0.05, p = 0.0002). Within 10 days of symptom onset, 3% of patients (n=3) were transferred to hospital due to clinical deterioration and 11% of patients (n=10) died. The result was followed for 30 days from the onset of symptoms in each patient.

Conclusion

Early treatment with DOXY for high-risk patients with moderate to severe COVID-19 infections in non-hospital settings, such as LTCFs, is associated with early clinical recovery, decreased hospitalization, and decreased mortality.

Categories: Infectious Disease
Keywords: covid-19, doxycycline, treatment, long-term care facility

Introduction

The COVID-19 pandemic placed an unprecedented and overwhelming burden on the U.S. healthcare system. Since the first case of COVID-19 in the U.S., there have been over two million COVID-19 cases and 122,000 deaths as of June 25, 2020 [1]. One-third of these deaths are nursing home residents or workers [2]. Data from small studies conducted in China [3] and France [4] present the clinical outcomes of COVID-19 patients who were treated with hydroxychloroquine (HCQ) and/or azithromycin (AZI). However, since HCQ and AZI are both cardiotoxic, there are concerns about the development of arrhythmia in patients treated with these drugs [5]. With limited treatment options, our group first treated high-risk COVID-19 patients in long-term care facilities (LTCFs) with doxycycline (DOXY) and HCQ [6]. DOXY has anti-inflammatory [7], immunomodulating [8], cardioprotective [9-10], and antiviral [11] activities. Subsequently, other studies noted the harmful side effects and mortality associated with HCQ administration [12].

Based on current evidence and reference articles that detail COVID-19 pathophysiology and DOXY's mechanisms of action, DOXY may be an effective drug in the treatment of COVID-19. As a result, we began treating high-risk patients with only DOXY and supportive care. The

efficacy of early DOXY intervention in high-risk COVID-19 patients in LTCFs is unknown. In this observational study, we collected data retrospectively, and we are presenting our clinical observations and outcomes of DOXY for high-risk patients with moderate to severe COVID-19 infections in LTCFs.

Materials And Methods

This case series assessed the clinical outcomes of eighty-nine (89) high-risk patients in LTCFs who developed a sudden onset of fever, cough, shortness of breath (SOB), and hypoxia between March 18 and May 13, 2020. High-risk patients were defined as patients who had at least one comorbidity such as hypertension (HTN), diabetes, coronary artery disease (CAD), congestive heart failure (CHF), chronic obstructive pulmonary disorder (COPD), obesity, or ventilator-dependency. These patients were diagnosed with COVID-19 and they were treated with an early course of DOXY (100 mg PO or IV for seven days) and regular standard of care. Since all patients were closely monitored by healthcare workers, COVID-19 symptoms were immediately noticed; DOXY was started within 12 hours and RT-PCR COVID-19 swab was collected. Assessed measures were patients' characteristics, fever, SOB, cough, oxygen saturation/pulse oximetry (POX), radiologic improvements, laboratory tests, DOXY side effects, hospital transfers, and death. Clinical recovery was defined as a resolution of fever, resolution of SOB, and improvement of POX (⩾93%). Follow-up chest X-rays (CXR) were ordered if clinically indicated.

The statistical analysis was done using Microsoft Excel 2016 (Microsoft Corporation, Redmond, Washington) with statistical application packages and IBM SPSS v23 (IBM Corp., Armonk, NY). The two-tailed T-test was used for between-group intervals in parametric data (e.g. POX). Binary logistic analysis and regression were used to assess the association of risk factors with the outcome. The patients' outcomes were recorded 30 days after symptoms onset.

All patients or patients' families gave informed consent before DOXY treatment was started, and oversight medical boards and corporate clinical services approved this observational study.

Results

Table *1* summarizes the characteristics, clinical features, lab and radiology results, outcomes, and side effects of DOXY of all 89 patients who were started with early DOXY treatment. The median age is 78 years, and the range is 43-101 years. One hundred percent (100%) of patients (n=89) tested positive for COVID-19 through reverse transcription-polymerase chain reaction (RT-PCR) and 85% of patients (n=76) showed clinical recovery. Eleven percent (11%) of patients died (n=10) and 3% of patients (n=3) were transferred to hospitals due to clinical deterioration.

Assessed Measures	LTCF Residents (n=89)	Percentage
Median Age, Years (Range)	78 (43-101)	
Male	50	56%
Female	39	44%
Clinically Recovered*	76	85%
Transferred to Hospital	3	3%
Death	10	11%
Hypertension (HTN)	62	70%

Diabetes	30	34%
Coronary Artery Disease (CAD)	41	46%
Congestive Heart Failure (CHF)	23	26%
Chronic Obstructive Pulmonary Disease (COPD)	31	35%
Obesity	37	42%
Ventilator Dependency**	4	4%
Fever	87	98%
Shortness of Breath (SOB)	85	96%
Cough	83	93%
Malaise/Weakness	71	80%
Altered Mental Status	56	63%
Diarrhea	31	35%
Chest Pain	27	30%
Tested COVID-19 Positive via RT-PCR	89	100%
High BUN	50	56%
High CR	46	52%
High LFT	33	37%
High Regular CRP	65	73%
High Ferritin	63	71%
High LDH	34	38%
High D-Dimer	57	64%
High Troponin	40	45%
High Procalcitonin	34	38%
Chest X-Ray (CXR) with Pneumonia	85	96%
Chest X-Ray (CXR) Improved	39	44%
Chest X-ray (CXR) Not Improved	41	46%
Chest X-Ray (CXR) Unavailable***	9	10%
Nausea, Vomiting, and Abdominal Discomfort	6	7%
No Side Effects	83	93%
Other Antibiotics	11	12%

TABLE 1: Characteristics, clinical outcomes, clinical features, lab and radiology results, side effects of doxycycline, and other antibiotics of all 89 high-risk COVID-19 patients who started seven-day doxycycline treatment in long-term care facilities

*Clinical recovery defined by resolution of fever (3.7 days), resolution of SOB (4.2 days), and average POX improvement 95%

**Four percent (n=4) of ventilator-dependent patients recovered and the ventilator setting returned to baseline.

***Chest X-ray not ordered, not required, or pending.

BUN: blood urea nitrogen; CRP: C-reactive protein; LFT: liver function test; LDH: lactate dehydrogenase; POX: pulse oximetry

Table *2* summarizes the characteristics, clinical features, lab and radiology results, outcomes, and side effects of DOXY of the remaining 76 patients who successfully completed the seven-day DOXY treatment. One hundred percent (100%) of patients demonstrated clinical improvement. After DOXY treatment started, resolution of fever and SOB occurred at an average of 3.7 days and 4.2 days, respectively. Average POX before and after treatment was 84% and 95%, respectively.

Assessed Measures	LTCF Residents (n=76)	Percentage
Median Age, Years (Range)	79 (43-101)	
Male	42	55%
Female	34	45%
Clinically Recovered*	76	100%
Hypertension (HTN)	52	68%
Diabetes	25	33%
Coronary Artery Disease (CAD)	35	46%
Congestive Heart Failure (CHF)	19	25%
Chronic Obstructive Pulmonary Disease (COPD)	26	34%
Obesity	33	43%
Ventilator Dependency**	4	4%
Fever	74	97%
Shortness of Breath (SOB)	72	95%
Cough	71	93%
Chest Pain	21	28%
Malaise/Weakness	62	82%
Altered Mental Status	45	59%

Diarrhea	25	33%
COVID-19 Positive	76	100%
High BUN	39	51%
High CR	39	51%
High LFT	26	34%
High Regular CRP	53	70%
High Ferritin	53	70%
High LDH	27	36%
High D-Dimer	48	63%
High Troponin	32	42%
High Procalcitonin	28	37%
Individuals with Pulse Oximetry (POX) Between 60% and 69% Before Treatment	4	5%
Individuals with Pulse Oximetry (POX) Between 70% and 80% Before Treatment	18	20%
Individuals with Pulse Oximetry (POX) Between 81% and 89% Before Treatment	42	47%
Individuals with Pulse Oximetry (POX) Between 90% and 92% Before Treatment	25	28%
Average Pulse Oximetry (POX) After Treatment	76	95%
Chest X-Ray (CXR) with Pneumonia	72	95%
Chest X-Ray (CXR) Improved	36	47%
Chest X-Ray (CXR) Not Improved	31	41%
Chest X-Ray (CXR) Unavailable***	9	12%
Nausea, Vomiting and Abdominal Discomfort	6	8%
No Side Effects	70	92%
Other Antibiotics	6	8%

TABLE 2: Characteristics, clinical outcomes, clinical features, lab and radiology results, side effects of doxycycline, and other antibiotics of 76 high-risk COVID-19 patients who completed seven-day doxycycline treatment in long-term care facilities

This table includes all patients who completed a full course (7 days) of DOXY therapy and excludes any patients who died and who were transferred to hospital and did not complete the seven-day course.

*Clinical recovery defined by resolution of fever (3.7 days), resolution of SOB (4.2 days), and average POX improvement 95% after treatment.

**Four percent (n=4) ventilator-dependent patients recovered and the ventilator setting returned to baseline.

***Chest X-ray either not ordered, or not required or pending.

BUN: blood urea nitrogen; CRP: C-reactive protein; LFT: liver function test; LDH: lactate dehydrogenase; POX: pulse oximetry

There was a statistically significant difference in POX before (pre-POX) and after (post-POX) treatment (n = 89, 84.7 ± 7% vs. 95 ± 2.6%, p = 0.0001). In the multivariate analysis model, the resolution of fever was associated with a reduction in mortality (Coeff = -0.96, p = 0.0001). Higher pre-POX and post-POX were also associated with decreased mortality; lower pre- and post-treatment POX is associated with increased mortality (pre-oxygen saturation (Spo2) vs Death, Coeff = -0.01, p = 0.023; Post-spo2 vs Death, Coeff = -0.05, p = 0.0002). Other variables, such as initial CXR with pneumonia, BUN, creatinine, liver enzyme levels, CRP, ferritin, LDH, D-Dimer, troponin, and procalcitonin, were not associated with mortality. Overall mortality at 30 days in our cohort was 11% (n=10). Clinical improvement was noted in 76 patients. Additionally, 3% (n=3) required transfer to a hospital while the rest of the patients were treated in the nursing home facility.

Discussion

COVID-19 is a positive-sense, single-stranded ribonucleic acid (RNA) virus [13], with a lipophilic outer shell that allows the pathogen to easily infiltrate lung tissue [14]. The coronavirus is believed to recognize and bind to cluster of differentiation 26 (CD26)/dipeptidyl peptidase-4 (DPP4) markers expressed on the cell surface, serving as an entry point for viral invasion [15]. COVID-19 infects the upper and lower respiratory tract, causing the release of pro-inflammatory cytokines such as interleukin 1β (IL-1β), tumor necrosis factor (TNF), and IL-6 [16]. COVID-19 induces mast cell proliferation within the respiratory submucosa [17] and activates the NF-kB pathway [18], further increasing the inflammatory response. As a result, individuals with COVID-19 present with fever, cough, and SOB. Severe infection can progress to acute respiratory failure and vascular thrombosis. COVID-19 is additionally correlated with acute cardiac injury and increased troponin I levels [19].

DOXY, an analog of tetracycline, is lipophilic and can also easily penetrate the lung epithelium [20]. Chemically modified tetracyclines (CMTs) can also induce apoptosis of mast cells [21]. The drug downregulates inflammatory markers demonstrated to have a role in COVID-19 pathophysiology. Evidence shows that DOXY downregulates the expression of DPP4 through the inhibition of the NF-kB pathway [22], impeding the virus's ability to enter cells and consequently decreasing the viral load. Along with its anti-inflammatory and immunomodulatory properties, DOXY is considered to be cardioprotective. During reperfusion after myocardial injury, matrix metalloproteinases-2 (MMP-2) are released. DOXY's inhibition of the MMP-2 pathway rescues left ventricular function [23]. As a result, in patients with acute ST-segment elevation myocardial infarct (STEMI) and left ventricular dysfunction, DOXY reduces adverse left ventricular remodeling [24]. DOXY's cardioprotective characteristics may improve clinical recovery in COVID-19 patients with acute myocardial injury.

DOXY is also thought to have antiviral activity. In-vitro assays of cultured cells inoculated with the dengue virus suggest that DOXY inhibits the virus's serine protease, disrupting viral replication, and viral entry into cells [25]. Another study demonstrates that when cells infected with murine retrovirus were treated with DOXY, there was a 70% decrease in retroviral load [26]. Tetracyclines can potentially treat other viral infections as well, such as viral encephalitis, West Nile virus, and human immunodeficiency virus (HIV) [27]. Further research reveals that elevated levels of intracellular zinc inhibit viral replication of COVID-19 [28] and that DOXY facilitates this inhibition by acting as a zinc ionophore, transporting, and increasing intracellular concentrations of zinc. Recently, a study demonstrated that in-vitro, DOXY itself

has antiviral activity against COVID-19 at a concentration of 5.6 µM [29].

DOXY has anti-inflammatory, immunomodulatory, cardioprotective, and antiviral properties. No observational study has yet been done using an early regimen of DOXY to treat moderate to severe high-risk COVID-19 patients in an LTCF. This case series demonstrates that early DOXY treatment in a non-hospital environment has good clinical recovery (85%, n=76), minimized hospital transfers (3%, n=3), and reduced death (11%, n=10).

A limit to this study is that there was no control group. We thus compared the data from this population to that of a similar reference patient population in an LTCF in Washington. This study performed an epidemiological investigation regarding 129 confirmed cases of COVID-19. Of the 129 confirmed cases, 63% (n=81) were LTCF residents (median age = 81 years, range = 54-100 years), 26% (n=34) were healthcare workers (median age = 42.5 years, range = 22-79 years, and 11% (n=14) were visitors (median age = 62.5 years, range = 52-88 years). Fifty-six point eight (56.8%) LTCF residents, 35.7% visitors, and 5.9% of healthcare personnel were hospitalized. Comorbidities among LTCF residents included HTN (69.1%), cardiac disease (56.8%), diabetes (37%), obesity (33%), and pulmonary disease (32.1%). Of the total population, 65.1% (n=84) were women. While no deaths occurred among staff members, 27.2% of LTCF residents and 7.1% of visitors died [30]. Comparing this study's data to our data using naive indirect comparison, early DOXY treatment may reduce hospital transfers and decrease mortality.

Conclusions

In this cohort of high-risk LTCF residents with moderate to severe COVID-19 infections who were treated with an early course of DOXY, improvement in oxygen saturation and resolution of fever were factors associated with lower mortality. The median time of all deaths in these high-risk patients was within the first 10 days in the course of illness. In the majority of our cohort, early initiation of DOXY treatment was associated with improved clinical outcomes, decreased hospitalizations, and decreased mortality. Larger randomized control trials are required to study the effect of DOXY treatment in moderate to severe COVID-19 infections.

Additional Information

Disclosures

Human subjects: Consent was obtained by all participants in this study. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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FOI 26-3132 - Document 5

TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE (1999) 93, 189–194

The effects of high-dose ivermectin regimens on *Onchocerca volvulus* in onchocerciasis patients

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Abstract

Ivermectin, at the standard dose of 150 μg/kg bodyweight, does not kill the adult worms of *Onchocerca volvulus* and does not disrupt embryogenesis or spermatogenesis. Repeated standard doses, if maintained, arrest microfilarial production but result in only a mild-to-modest macrofilaricidal effect. We investigated whether high doses would effectively kill the adult worms, and whether cessation of microfilarial production could be reproduced by an equivalent, single, high dose. One hundred men participated in a double-blind placebo-controlled trial and received increasing doses of ivermectin from 150 μg/kg to 1600 μg/kg bodyweight. Nodules were excised at day 180 and examined by histopathology. Total doses of ivermectin up to 1600 μg/kg were not significantly more effective than 150 μg/kg. Moreover, they did not reproduce the marked inhibitory effects of the repeat standard-dose regimens on embryogenesis, nor the modest effect on adult worm viability, at comparable total doses. These effects may be functions of multiplicities of dosages rather than of the total dose. Our findings also suggest that repeated high-dose regimens are unlikely to be more effective than a similar number of 150 μg/kg doses. This deficiency of ivermectin requires that the search for macrofilaricides remains a top priority.

Keywords: onchocerciasis, *Onchocerca volvulus*, chemotherapy, ivermectin, efficacy, drug regimens, adult worms

Introduction

Ivermectin is the drug of choice for the treatment of onchocerciasis. When given at the standard dose of 150 μg/kg bodyweight it does not kill the adult worms and does not disrupt embryogenesis or spermatogenesis. The main effects are a potent microfilaricidal action and a block to the release of stretched microfilariae (mf) from the adult female worms followed by their degeneration *in utero* (WHO, 1995).

Initial attempts to investigate the macrofilaricidal potential of ivermectin have led to the repeated administration of 100–150 μg/kg bodyweight. These dose regimens have varied in frequency and duration and total doses have ranged from 600 μg/kg to 1800 μg/kg bodyweight. Indirect evidence of the effect on the adult worms obtained by studying the kinetics of skin mf numbers showed that there was a permanent decline in mf production (GREENE *et al.*, 1991). This has been quantified, using model calculations, at ~30% per treatment (PLAISIER *et al.*, 1995).

Other investigators have defined the nature of this decline directly by examining the adult worms. A regimen of 100 μg/kg bodyweight given every 2 weeks to a total dose 600 μg/kg bodyweight had no adulticidal effect (DUKE *et al.*, 1991a). Three studies in Guatemala investigated the effects of 150 μg/kg given monthly for 4, 8 or 12 doses (Guatemala I), as a single dose compared with 4 doses given every 6 months (Guatemala II), and (in Guatemala III) as 4, 8 or 11 doses given every 3 months (DUKE *et al.*, 1990, 1991b, 1992). The major effect of these regimens was a reduction in the proportion of female worms producing mf. The effect was most marked in Guatemala III where mf production ceased 4 months after 8 or 11 doses. Other findings included an increase in the proportion of unfertilized or uninseminated worms and of female worms with degenerate mf. There were fewer male worms per nodule and an increase in the number of nodules without male worms in Guatemala I and Guatemala III. However, macrofilaricidal activity was not impressive. A mild increase in both adult male and female worm mortality (12% and 22%, respectively) occurred only with 12 monthly doses (total dose 1800 μg/kg). A modest increase in mortality among female worms occurred in groups given 8 (25·5%) or 11 (32·6%) 3-monthly doses (total dose 1200 μg/kg or 1650 μg/kg).

In Sierra Leone, 4 × 150 μg/kg and 5 × 150 μg/kg bodyweight of ivermectin (given 6-monthly to a total dose of 600–750 μg/kg) were compared with 150 μg/kg and placebo (CHAVASSE *et al.*, 1992). A single dose of ivermectin did not affect embryogenesis and produced many degenerating mf. Multiple doses severely depressed embryogenesis. There was a significant trend with increasing doses towards fewer worms with multicellular embryonic stages and stretched mf and there were fewer gravid worms. Oocytes were produced normally but there was an increase in the proportion of abnormal forms with dose. Embryogenesis was arrested at the single-cell stage possibly because of reduced fertilization. A probable cause for this was ineffective insemination of the female worms as shown by the much lower proportion of seminal receptacles containing sperm (CHAVASSE *et al.*, 1993). There was no significant reduction in the number of live male worms per nodule.

The repeat standard-dose regimens have shown that ivermectin given 3- or 6-monthly to a total dose of ⩾600 μg/kg markedly suppressed mf production and produced minor to modest macrofilaricidal effects at total doses of ⩾1200 μg/kg bodyweight.

An important consideration in the selection of repeat standard-dose regimens in the investigation of the macrofilaricidal potential of ivermectin was the lack of information on the safety of the drug at higher doses. In an initial investigation, we reported the safety of single, increasing doses of ivermectin from 150 μg/kg through 800 μg/kg bodyweight given with or without a 'clearing' dose of 150 μg/kg bodyweight (AWADZI *et al.*, 1995). This study was eventually extended to a total dose of 1600 μg/kg bodyweight. The aim was to determine whether high doses of ivermectin effectively kill the adult worms. An additional interest was whether the marked effects on embryogenesis achieved by 5 to 12 repeated standard-dose regimens could be duplicated or augmented by an equivalent, single, high dose. This paper compares the parasitological effects of the standard- and high-dose regimens.

Patients and methods

Patients were healthy men, infected with *Onchocerca volvulus*, from the forest region of south-eastern Ghana. The study took place between January 1994 and October 1995 at the Onchocerciasis Chemotherapy Research

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The effects of high-dose ivermectin regimens on Onchocerca volvulus in onchocerciasis patients 16/3/2023, 5:53 pm
FOI 26-3132 - Document 5

Centre (OCRC) at Hohoe (Ghana) located 80 km south of the project area. Vector control measures had begun in the area 7 years earlier and transmission of onchocerciasis had been interrupted. Verbal consent was obtained from each patient after all the procedures involved had been explained in detail. The study was approved by the Ethics Committee of the Ministry of Health, Ghana, and the WHO Secretariat Committee for Research Involving Human Subjects (SCRIHS).

The details of the conduct of this randomized, double-blind, placebo-controlled trial and the clinical and laboratory safety aspects have been presented previously (AWADZI *et al.*, 1995). During this phase 75 patients were treated. In brief, systemic clinical, ophthalmological and laboratory tests were conducted before, during and after drug administration. Ophthalmological examinations included perimetry, slit-lamp examination of the anterior segment, colour photography and fluorescein angiography. Systemic and ocular reactions to treatment were quantified (AWADZI, 1980; HERO *et al.*, 1992). Laboratory tests included the determination of mf densities in skin snips and standard biochemical and haematological tests. Ivermectin or matching placebo tablets were administered on days 1 and 4; ivermectin dosages were increased in successive groups of patients from 150 μg/kg to 800 μg/kg. With the high-dose regimens some patients received a 'clearing' dose of 150 μg/kg on day 1 followed by the high dose on day 4 while others received the high dose on day 1 followed by placebo on day 4. Clinical and laboratory tolerances were determined on data collected through day 30.

Besides the 75 patients reported on previously there were 6 other patients included as controls for a study of the immunopathogenesis of the Mazzotti reaction carried out in parallel over the first 72 h; they received placebo tablets on day 1 followed by 150 μg/kg of ivermectin on day 4. Subsequently, 19 additional patients were treated as a single group to bring the total number enrolled to 100. Nine of these patients received 800 μg/kg of ivermectin as a single dose and 12 others received the same dose repeated after 13 days. There was no reaction to the second 800 μg/kg dose. Follow-up for all patients took place on days 30, 90, 180 and 365 when all clinical and laboratory examinations were repeated. At day 180, all palpable nodules were aseptically excised using 1% xylocaine. Some nodules were examined using in-vitro tests (BENNETT & PAX, 1986; COMLEY *et al.*, 1988; STROTE *et al.*, 1993) while the rest were processed for histopathology. Nodules for histology were freed from excess host tissue, washed in saline and fixed in 4% (w/v) buffered formaldehyde and embedded in paraffin. From each nodule in the plane of the largest diameter, at least 10 sections of 4-μm thicknesses were cut, stained with haematoxylin and eosin and examined by microscopy. Adult worm viability and reproductive activity were assessed as described previously (BUTTNER *et al.*, 1988).

Statistical analysis

The treatment groups were ordered according to the total dose of ivermectin received. The distribution of nodule characteristics, adult worm viability and of female worms in various reproductive stages were compared using the χ^2 and trend tests. Microfilarial counts were logarithmically transformed before analysis. Skin mf counts are the adapted sums of the densities at the iliac crests and calves. Ocular counts are the adapted sums for both eyes. The means of these counts were compared using analysis of variance (ANOVA); comparison of group means was done where significant differences occurred using the t test and pooled SD. The kinetics of skin mf numbers in each group was studied by comparing the mean counts of adjacent visits (paired t test). The null hypothesis was rejected when P was less than 0·05.

Results

Categorization of patient groups

One patient could not be traced after the day 180 follow-up and was eliminated from the study. The data presented relate to the 99 patients who were available at all time points up to day 365. Owing to the numbers of patients in the individual treatment groups whose nodules had been examined by histology, it was necessary to combine the groups in a meaningful way to permit reasonable statistical analyses to be undertaken. The final allocation of patients created 5 treatment groups (based on total dose range) as follows:

Group 1. 150 μg/kg (control);
Group 2. 400 μg/kg preceded by 150 μg/kg or placebo (400–550 μg/kg);
Group 3. 600 μg/kg preceded by 150 μg/kg or placebo (600–750 μg/kg);
Group 4. 800 μg/kg preceded by 150 μg/kg or placebo (800–950 μg/kg); and
Group 5. 800 μg/kg preceded by 800 μg/kg (1600 μg/kg).

Nodule characteristics and adult worm viability

The results presented are of those nodules examined by the standard method of histopathology. Data from tests *in vitro* are not presented as the method is still being assessed as an alternative to histopathology. A total of 225 nodules from 60 patients were examined. These yielded 495 adult female and 190 adult male worms. The numbers of nodule donors and of the nodules examined, the basic characteristics of the nodules and the effect on adult worm viability for each treatment group are shown in Table 1.

There were no significant trends regarding the number of live female or male worms per nodule, the male:female ratio or in the number of nodules with live mf. There was however a significant trend to a reduction in the number of nodules without male worms with increasing doses of ivermectin (χ^2 for trend = 5·78; $P = 0·02$). With increasing doses of ivermectin, there was no significant trend in mortality among female and male worms in the treated groups. Overall, 32·7% of all female worms and 9·5% of all male worms were dead/moribund and 17·0% of the female worms were calcified.

Among 255 female worms obtained from 107 nodules excised from 25 untreated patients at the beginning of vector control in south-eastern Ghana, 26% were dead/probably dead and 12% were calcified while 4% of 79 male worms were dead/probably dead (BUTTNER *et al.*, 1990). Although there were increases in the proportions of dead and calcified adult worms in this study population as compared with pre-control data, none of the differences was significant ($P > 0·05$ for all comparisons). Thus, 7 years of successful vector control had not invalidated the suitability of the adult worm population for clinical trials. An unusual finding that was probably unrelated to therapy was the tendency to a reduction in the proportion of calcified adult female worms with increasing doses of ivermectin.

Adult worm reproductive activity

The number and distribution of female worms in different reproductive stages are shown in Table 2. The main findings were an increase in the proportion of female worms with nearly empty uteri, and a reduction in the proportion with young embryos and coiled mf with increasing doses of ivermectin. There was no significant trend with dose in the proportion of worms with young oocytes only, with stretched mf, or with degenerate stretched mf. There was no marked effect on embryogenesis. Between 95% and 100% of live male worms contained intact spermatozoa with no differences between the groups (data not shown).

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The effects of high-dose ivermectin regimens on Onchocerca volvulus in onchocerciasis patients

FOI 26-3132 - Document 5

16/3/2023, 5:53 pm

Table 1. Nodule donors, nodule characteristics and the effects of standard- and high-dose ivermectin on adult *O. volvulus* worm viability

Treatment group and ivermectin dosage	No. of nodule donors	No. of nodules	Nodules without male worms No. (%)	Nodules with live mf No. (%)	Female worms			Male worms	
					All	Dead or probably dead No. (%)	Calcified No. (%)	All	Dead or probably dead No. (%)
150 µg/kg	13	63	40 (63·5)	13 (20·6)	132	50 (37·9)	30 (22·7)	36	3 (8·3)
400–550 µg/kg	12	60	29 (48·3)	6 (10·0)	130	42 (32·3)	25 (19·2)	52	4 (7.7)
600–750 µg/kg	12	49	19 (38·8)	4 (8·2)	87	20 (23·0)	10 (11·5)	39	3 (7·7)
800–950 µg/kg	14	32	13 (40·6)	8 (25·0)	89	25 (28·1)	10 (11·2)	36	3 (8·3)
1600 µg/kg	9	21	9 (42·9)	2 (9·5)	57	25 (43·9)	9 (15·8)	27	5 (18·5)
Total	60	225	110 (48·9)	33 (14·7)	495	162 (32·7)	84 (17·0)	190	18 (9·5)
χ^2 for trend			5·783	0·273		0·101	4·751		1·344
P values			0·016	0·601		0·75	0·03		0·246

FOI 26-3132 - Document 5

Table 2. The effects of standard- and high-dose ivermectin on adult *O. volvulus* female worm reproductive activity

Treatment group and ivermectin dosage	All	Live female worms: With nearly empty uteri No. (%)	With oocytes only No. (%)	With young embryos No. (%)	With coiled mf No. (%)	With stretched mf No. (%)	With degenerate stretched mf[a] No. (%)
150 µg/kg	82	14 (17·1)	24 (29·3)	26 (31·7)	26 (31·7)	38 (46·3)	30 (78·9)
400–550 µg/kg	88	20 (22·7)	14 (15·9)	27 (30·7)	25 (28·4)	53 (60·2)	50 (94·3)
600–750 µg/kg	67	18 (26·9)	20 (29·9)	9 (13·4)	10 (14·9)	27 (40·3)	27 (100.0)
800–950 µg/kg	64	17 (26·6)	13 (20·3)	14 (21·9)	15 (23·4)	31 (48·4)	28 (90·3)
1600 µg/kg	32	11 (34·4)	4 (12·5)	5 (15·6)	2 (6·2)	17 (53·1)	15 (88·2)
Total	333	80 (24·0)	75 (22·5)	81 (24·3)	78 (23·4)	166 (49·8)	150 (90·4)
χ^2 for trend		4·202	1·738	5·947	8·295	0·034	0·107
P values		0·04	0·187	0·015	0·004	0·854	0·744

[a]Of those with stretched mf.

Skin and ocular microfilariae

The skin and ocular mf counts over 1 year are shown in Table 3. An earlier presentation summarized the results at days 3, 7 and 11 (AWADZI *et al.*, 1995). Within each group there was a rapid reduction in counts reaching a nadir at day 30. This value was maintained over the next 3 months after which successive increases occurred at days 180 and 365. This pattern of kinetics of skin mf numbers reflects an initial potent microfilaricidal effect and a subsequent recovery from the effects of the block to mf release. Comparison of the group geometric means revealed no significant differences initially, at day 90 or at day 365. The groups were however significantly different at days 30 and 180. At day 30 the differences were due to a greater reduction in 3 of the 4 high-dose groups (namely Groups 2, 3 and 5) when compared to the 150 µg/kg group; the 800–950 µg/kg group was the exception. At day 180, only the 2 highest dose groups (800–950 µg/kg and 1600 µg/kg) produced greater reductions than the 150 µg/kg group. These groups appeared to have a greater suppression of skin mf at this time than the lower dose groups but the effect was minor (maximum difference 1·6% reduction) and transient (lasted less than 6 months) and by 1 year the mean skin mf densities were again similar in all groups.

Sixty-three of 99 patients had mf in the anterior chamber of the eye. The counts were highly variable between patients (1–86 mf in both eyes). The initial group geometric mean counts were low and varied from mean (95% confidence interval) 1·3 (0·2–3·5) to 3·5 (1·7–6·7). Comparison of the group geometric means revealed no significant differences at any time. The data for all the groups were therefore pooled. Within each group there was a delay in reduction of mf counts till day 30 or 90. Small numbers of mf (2–11) persisted in 9 patients at 1 year. Overall, the treatment groups maintained at 1 year a 96% reduction on initial counts for both skin and ocular mf.

Discussion

The 'ideal' drug for the treatment and control of onchocerciasis should be safe, macrofilaricidal, effective in a single dose, uniformly effective in all endemic areas, cheap and stable. It should readily merge into existing health care delivery strategies and be compatible with drugs already in use for common endemic diseases. Ivermectin would have satisfied all these requirements but for the lack of a potent macrofilaricidal effect. This potential was explored in many studies that relied on giving repeat standard-dose regimens. The dominant effect of these regimens was however on the reproductive activity of the female worms. The effects observed include an increase in the number of females with unfertilized ova, depression of the rate of new embryonic development, a decrease in the proportion of females producing mf, a decrease in the number with degenerate mf and a cessation of microfilarial production. An important explanation for these findings was a failure of insemination of the female worms. Among the mechanisms suggested are a reduction in the number of nodules with male worms, an abnormality of spermatogenesis, a disinclination of the male worms to mate, a block to the passage of sperm by mf degenerating in the uteri and impairment of oogenesis.

Although not the ideal situation, a permanent sterilization of the adult worms, or even a prolonged suppression of mf production over many years, could form a rational basis for a control programme pending the coming of the ideal drug. So far, a sustainable suppression of mf production has depended on multiple dosing with ivermectin over several years (KLÄGER *et al.*, 1996).

One rationale for our study was that, if the total dose administered was the major factor in the embryo suppression characteristic of the repeat-dose regimens, then the administration of a safe, single high dose that achieved similar effects would greatly simplify the use of ivermectin in the control of onchocerciasis. The preferred outcome, however, was that the high dose would kill the adult worms.

In order to assess the effects on the adult worms, pooling of the data was necessary owing to the number of patients providing nodules for histopathology in the individual groups. Pooling involved the combination of those who had received a 'clearing' dose of 150 µg/kg with those who had not with respect to a given high dose. Since this dose has no macrofilaricidal activity, it is unlikely that results of adult worm viability would be unduly influenced by this procedure. Other related data (skin mf densities, ocular mf counts) were similarly regrouped to permit parallel comparisons to be made.

There was no increase in mortality among the adult worms with increased dosage with ivermectin. In all groups there were worms in various stages of reproductive activity and included worms with multicellular stages and stretched mf. Although there was a significant trend towards an increase in the number of female worms with nearly empty uteri and a reduction in the proportion of female worms with young embryos and coiled mf with increasing dose, this did not materially affect the production of stretched mf.

An indirect assessment of the effects on the adult worms was obtained by a study of the kinetics of skin mf numbers over time. This revealed differences between the groups only at day 30 and day 180. At day 30 the 150 µg/kg group was at a slight disadvantage as 6 of the members had acted as controls for the Mazzotti reaction and were treated 3 days after the other patients. On the other hand the 1600 µg/kg group (800 µg/kg × 2) had a slight advantage in that the second dose of 800 µg/kg was given on day 13 while the other groups had completed

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Table 3. The effects of standard- and high-dose ivermectin on skin and ocular *O. volvulus* microfilariae

Treatment group and ivermectin dosage	Initial count (GM)[a]	Time after chemotherapy							
		Day 30		Day 90		Day 180		Day 365	
		GM	(% Reduction)	GM	(% Reduction)	GM	(% Reduction)	GM	(% Reduction)
150 µg/kg ($n = 15$)	192 (146–253)	0·5 (0·2–0·9)	(99·7)	0·6 (0·06–1·4)	(99·7)	4 (2·3–6·8)	(97·9)	12·4 (7·0–21·5)	(93·5)
400–550 µg/kg ($n = 25$)	172 (133–224)	0·2 (0·03–0·36)	(99·9)	0·2 (0·03–0·30)	(99·9)	2·5 (1·6–3·9)	(98·5)	6·1 (3·2–10·9)	(96·4)
600–750 µg/kg ($n = 23$)	210 (164–270)	0·07 (–0·01–0·16)	(>99·9)	0·1 (–0·02–0·30)	(99·9)	3·7 (2·2–5·9)	(98·2)	8·0 (4·0–14·8)	(96·2)
800–950 µg/kg ($n = 24$)	203 (156–265)	0·3 (0·07–0·56)	(99·8)	0·3 (0·08–0·51)	(99·9)	1·5 (0·8–2·5)	(99·3)	6·0 (3·2–10·7)	(97)
1600 µg/kg ($n = 12$)	172 (124–237)	0	(100)	0	(100)	0·8 (0·4–1·4)	(99·5)	4·4 (1·5–10·6)	(97·4)
P values[b]	0·65	0·02		>0·05		<0·01		0·37	
Pooled mfac[c] ($n = 99$)	2·7 (1·8–3·8)	0·9 (0·6–1·3)	(66·7)	0·4 (0·2–0·6)	(85·2)	0·2 (0·08–0·28)	(92·6)	0·1 (0·04–0·24)	(96·3)

[a]GM = geometric mean (95% CI in parentheses).
[b]Based on the comparison of the geometric means of all treatment groups (analysis of variance).
[c]Counts of microfilariae in the anterior chamber of the eye were combined for all treatment groups.

The effects of high-dose ivermectin regimens on Onchocerca volvulus in onchocerciasis patients
FOI 26-3132 - Document 5
16/3/2023, 5:53 pm

The effects of high-dose ivermectin regimens on Onchocerca volvulus in onchocerciasis patients 16/3/2023, 5:53 pm
FOI 26-3132 - Document 5

therapy on day 4. The findings at day 180 suggest a more complete clearance of mf with the highest doses, but the similarity of mf loads in all groups at 1 year indicates that the effect is of little importance. The clearance of ocular mf was also similar in all groups.

In summary, single doses of ivermectin as high as 800 μg/kg (more than 5 times the standard dose) or of 1600 μg/kg given in 2 doses (more than 10 times the standard dose) are as safe as the standard dose but are not clearly superior in their antiparasitic effects. Moreover, they do not reproduce the marked inhibitory effects of the repeat standard-dose regimens on embryogenesis, nor the mild-to-modest effect on adult worm viability, at comparable total doses. This suggests that it is the multiplicity of dosages rather than the total dose that is the factor that determines the effects. This study also reconfirms that the ceiling of therapeutic activity of ivermectin against *O. volvulus* is reached with 150 μg/kg (AWADZI *et al.*, 1985). A further extension of this assertion is that multiple high-dose regimens are unlikely to be more effective against the adult worms than a similar number of doses of the standard regimen. This deficiency of ivermectin requires that the search for macrofilaricides for onchocerciasis remains a top priority.

Acknowledgements

We are grateful to Merck Sharp & Dohme Research Laboratories for the supply of ivermectin and placebo tablets. This investigation received financial support from the African Programme for Onchocerciasis Control–Onchocerciasis Control Programme in West Africa–UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We are also grateful to Professor D. W. Büttner of the Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany, for nodule histological data that are generally in agreement with those presented in this paper.

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Annals of Tropical Medicine & Parasitology, Vol. 98, No. 3, 231–249 (2004) FOI 26-3132 - Document 5

An investigation of persistent microfilaridermias despite multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana

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Received 11 July 2003, Revised 5 January 2004, Accepted 7 January 2004

If ivermectin-based programmes for the control of human onchocerciasis are to be successful, the drug must remain effective for as long as necessary. In an open, case–control study, an attempt was made to determine if the persistent, significant, *Onchocerca volvulus* microfilaridermias seen in some individuals who had received at least nine treatments with ivermectin were the result of the development of drug resistance in the parasite. Twenty-one of these 'sub-optimal' responders (cases) were matched, by age, weight, number of treatments, locality and skin microfilarial counts, with seven amicrofilaridermic responders and 14 ivermectin-naive subjects. The number of treatments taken, any potential drug interactions and significant underlying disease were determined from detailed clinical and laboratory studies. Each subject was treated with ivermectin during the study, so that plasma concentrations of the drug could be determined for 72 h from the time of dosage. The microfilarial and adult-worm responses to this treatment were assessed from skin microfilarial counts (obtained before the treatment and at days 8, 90 and 365 post-treatment), day-90 embryogrammes, and the results of fly-feeding experiments. Parasite-sensitivity criteria for various time-points were derived from earlier data on skin microfilaridermias and the effects of ivermectin on the adult worms. The results indicate that the significant microfilaridermias that persist despite multiple treatments with ivermectin are mainly attributable to the non-response of the adult female worms and not to inadequate drug exposure or other factors. The possibility that some adult female worms have developed resistance to ivermectin cannot be excluded. These results justify the routine monitoring of treatment efficacy in any ivermectin-based programme of disease control.

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DOI: 10.1179/000349804225003253

FOI 26-3132 - Document 5

In onchocerciasis-endemic areas with ongoing transmission, the human host often harbours various stages of the filarial parasite *Onchocerca volvulus*, the causative agent of the disease. These include the infective larvae, the migrating and developing pre-adult forms, the adult male and female worms and the microfilariae (mff). Many adult worms are readily accessible in subcutaneous nodules (onchocercomata) whereas the millions of mff that they produce, and that cause most of the pathology in onchocerciasis, reside predominantly in the skin and eye. The infective third-stage larvae (L_3), the form transmitted by the blackfly (*Simulium*) vector to the human host, develop in the vector from the mff taken up from the skin. Human onchocerciasis has been a major cause of disfiguring skin lesions, visual impairment and blindness, with attendant socio–economic problems. The World Health Organization (WHO) estimated that $>99\%$ of the global totals of those infected and those blind as a result of the disease live in the tropical belt of Africa (WHO, 1995). Because of this, the Onchocerciasis Control Programme in West Africa (OCP) and the African Programme for Onchocerciasis Control (APOC) were set up, with the common aim of eliminating onchocerciasis as a disease of public-health importance (Webbe, 1992; Benton, 1998).

In the absence of a protective vaccine, the control of onchocerciasis depends on measures directed against the vector and on chemotherapy. The drugs (diethylcarbamazine and suramin) that were available at the inception of the OCP had serious drawbacks that limited their mass administration and use in public-health programmes for the control of onchocerciasis. Thus, initially, the OCP relied on control of the vector, using insecticides to attack the larval stages of blackflies in their aquatic habitat. With the introduction of ivermectin, however, an additional tool became available. Ivermectin is a safe drug that has a potent effect against the mff and also temporarily blocks release of the mff by the adult female worms. Since a single, first dose of 150–200 μg ivermectin/kg has no effect on the vitality or reproductive potential of the adult worms (WHO, 1995), the release of mff begins again post-treatment and mff gradually repopulate the skin. In order to keep the skin free of mff, repeated administration of ivermectin, one or more times a year, is therefore required. Unlike the first dose, repeated treatments with ivermectin impair the ability of the female worms to produce mff (Chavasse *et al.*, 1992; Duke *et al.*, 1992; Plaisier *et al.*, 1995; Klager *et al.*, 1996) and also have a variable effect on adult-worm vitality (Duke *et al.*, 1992; Gardon *et al.*, 2002). The combined effects of multiple treatments on the mff and the adult worms result in the disappearance of mff from the skin (Guderian *et al.*, 1997), a reduction in the uptake of mff by the vector (Chavasse and Davies, 1990; Cupp *et al.*, 1992), a reduction in transmission (Boussinesq *et al.*, 1997; Boatin *et al.*, 1998) and hence a reduction in the incidence of onchocerciasis (Boussinesq *et al.*, 1995).

In the control of onchocerciasis, ivermectin is used either as the sole agent or as an adjunct to vector control. If ivermectin-based programmes, such as the APOC, are to be successful, the drug must remain effective against the mff and the adult worms for as long as it takes to control or eliminate the disease. The worrying possibility that *O. volvulus* could develop resistance to ivermectin has been considered (Awadzi, 1993; Shoop, 1993; Boussinesq and Gardon, 1999; Dadzie *et al.*, 2003), and the WHO has urged that methods be established to detect the development of such resistance, and to study the underlying mechanisms (WHO, 1995).

In 1987, the OCP introduced large-scale distribution of ivermectin, beginning in the Pru and Lower Black Volta (LBV) river basins in Ghana. Vector control began in the LBV basin in 1975 and was stopped in 1996. In the Pru basin, vector control started in 1988 and continues to date. Field surveys carried out in 1997, at Chingakrom and

FOI 26-3132 - Document 5

Agbelekame I in the LBV basin and at Asubende, Faomang and Hiampe in the Pru River basin, revealed individuals with persistent, significant microfilaridermia (PSM). Others subjects from the same communities, who had similar microfilarial loads in 1987 and had subsequently been exposed to the same environmental factors and received an identical number of treatments to those found with PSM, were skin-snip-negative (unpubl. obs.). As a working, 'field' definition, individuals who still had at least 10 mff/snip in 1997, after nine or more treatments with ivermectin, were labelled as 'sub-optimal responders' (SOR) in 1997. Subjects who were snip-negative in 1997 although known to have been positive in 1987 were considered 'responders'. The potential factors that could result in sub-optimal response, and the possible approaches to their investigation, are summarized in Table 1.

The primary objective of the present investigation was to determine whether sub-optimal response was due to parasite factors. This involved the exclusion of subject- and pill-related factors and the assessment of the parasite response on re-challenge with ivermectin under standard, hospital conditions. In addition, parasite material was collected from sub-optimal responders, responders and a population not previously exposed to ivermectin, to determine, in a subsequent study, if genetic differences could account for any observed differences and provide a plausible explanation for the possibility that the mff and/or the adult female worms of *O. volvulus* have developed resistance to ivermectin.

SUBJECTS AND METHODS

Subject Selection and Study Design

Lists of potential study subjects were extracted from the OCP database in line with the working field definitions of SOR and responder. Overall, 26 SOR suitable as subjects — 12 males and five females from the LBV basin and nine males from the Pru basin — were identified. Their ages ranged from 15–67 years and they had had 14.5–213 mff/snip when last examined. In case an inadequate number of these SOR could be recruited, a 'standby list' of two male and two female subjects with counts of nine mff/snip was also created. 'Responders', who were matched for sex and approximate age (15–67 years) with the SOR, were

TABLE 1. *The possible reasons for sub-optimal responses to ivermectin, and their investigation*

Factor	Investigative approach
THE PILL	Stability, storage, transportation, distribution
THE SUBJECT	
Failure to consume drug	Detailed history of intake, adverse events to each dose, review of records
Adverse interactions with other drugs or herbs	Activities in temporal relationship to ivermectin intake
Inadequate drug exposure	Pharmacokinetic study
Underlying disease (immunosuppression)	Clinical and laboratory examinations
THE PARASITE	
Patent new infections	Review of epidemiological data. Evidence of exposure to intense transmission
Abnormal response (tolerance or resistance)	Response of microfilariae and adult worms on re-challenge, measured using skin snips, fly feeding, and/or embryogrammes. Demonstration of a plausible genetic basis, using individual parasites from multi-dosed responders, sub-optimal responders and the ivermectin-naive

FOI 26-3132 - Document 5

identified from the same localities: eight male and nine female subjects from the LBV basin and 19 male and six female subjects from the Pru basin. (A much higher number of responders than SOR was selected because it was assumed that those who were already snip-negative would be less likely to volunteer for the study, as many would derive no direct benefit from their participation.) In 2000, the Directorate for Onchocerciasis Control of the Ghanaian Ministry of Health located 28 subjects from the lists who were willing to participate in the study: 15 SOR and 13 responders. The study was an open, case–control investigation. The SOR (the cases) were matched, by age, body weight and number of treatments with responders from the same localities and with 14 subjects from the Tordzi river basin, an area without previous ivermectin distribution or vector control. The Tordzi subjects, henceforth referred to as the 'ivermectin-naive', had physical characteristics similar to those of the SOR and responders and levels of microfilaridermia that were broadly similar to those of the SOR.

Between May 2001 and July 2002, the subjects were treated with ivermectin in Hohoe Hospital, Hohoe, Ghana, so that post-treatment plasma concentrations of the drug and the responses of the subjects and their parasites to the treatment could be carefully monitored (see below). At the beginning of the hospital-based study (i.e. 3–4 years after the multi-dosed subjects had been categorized in the field as SOR or 'responders'), seven 'responders' were found to have become snip-positive and only a single microfilaria was found in snips taken from four sites on one of the SOR (who was subsequently skin-snip-negative). As a result of these observations, a working 'hospital' definition was developed, in which the SOR were the 21 subjects from the select lists who were snip-positive on admission (irrespective of the intensity of their microfilaridermia), and the seven subjects who were snip-negative on admission were termed 'responders'.

Ethical Aspects

In 2001, before screening procedures were initiated in the study villages, informed consent was obtained from each villager volunteering to be a subject. A document detailing the conduct of the study was available in English and two local languages. This was read out to the volunteers who, after exhaustive discussions, gave verbal consent to participate in the investigation. The process was documented, on a form for each subject, by a project technician or co-ordinator and by an impartial witness from the community. Each consent form was countersigned and dated by the principal investigator or a co-investigator. The Ghanaian Ministry of Health and the WHO Secretariat Committee for Research Involving Human Subjects approved the study protocol. The subsequent hospital-based study was conducted in compliance with good clinical practices and in accordance with the Declaration of Helsinki (2000 revision).

Determination of Baseline Clinical Characteristics

In 2001, while in their villages, the subjects had been asked about the number of ivermectin treatments taken, the adverse events to each dose and the use of any other drug or herbal preparation in the few days before or after ivermectin intake.

Later, while hospitalized in Hohoe, the subjects lived under standard conditions; they had a common diet and the use of alcohol, tobacco, herbal medications and non-prescribed drugs was banned. A more detailed medical history was taken from each subject and a general physical examination performed. The subjects' eyes were examined as described previously (Awadzi *et al.*, 1997), with the exclusion of fundus photography and fluorescein angiography. Haematocrits, haemoglobin concentrations, platelet and total leucocyte counts, absolute counts of granulocytes, lymphocytes and monocytes (and the related percentages of all the

FOI 26-3132 - Document 5

leucocytes) were recorded. Each subject was screened for glucose-6-phosphate-dehydrogenase (G6PDH) deficiency and his plasma concentrations of sodium, potassium, chloride, bicarbonate, alanine aminotransferase (SGOT), aspartate aminotransferase (SGPT), lactic dehydrogenase, gamma-glutamyl transferase, alkaline phosphatase, total and conjugated bilirubin, albumin, total protein, sugar, cholesterol and triglycerides were determined in a 'fasting' blood sample. Stool and urine samples were checked for helminth and other parasites, using Kato–Katz smears and filtration through Nucleopore® filters (Nucleopore, Pleasanton, CA), respectively.

Skin snips were taken shortly before the treatment. In the field and in the hospital, skin snips were taken from both iliac crests and both calves with a Walser-type corneo-scleral punch. Punches were sterilized between subjects, in an autoclave. Each snip was weighed and then incubated for approximately 24 h in isotonic saline in a flat-bottomed well of a microtitre plate. Any mff in the well were then counted on an inverted microscope. The microfilarial density at each snip site was expressed as the number of mff/mg skin, the count for each subject (mff/4 mg skin) being the sum of the densities (in mff/mg) at each of the iliac crests and each of the calves.

Determination of Baseline Entomological Indices

During the bite of an infected human by a female blackfly seeking a bloodmeal, mff are taken up from the dermis. The mff undergo temperature-dependent morphological changes, transforming into infective L_3 that may be deposited in the skin of another human during the next bloodmeal. Microfilariae cannot be taken up from the skin if they have been cleared by treatment, and ingested mff may not develop into infective larvae if they have been damaged or modified by the treatment. Thus, by allowing the appropriate blackflies to feed on trial subjects under controlled conditions before and after treatment, the sensitivity of the mff to the administered drug can be determined. The use of newly emerged flies ensures that any mff found in the fly could only have been taken up during the experiment.

For the present, hospital-based investigation, pupae of *S. damnosum* s.l. were collected, from breeding sites at Obosomase and Krobo on the River Pra, and reared to adults in emergence cages. On admission and before treatment, the legs of each subject were exposed to 30 newly emerged female blackflies, which were allowed to feed undisturbed for 30 min. Some of the blood-fed flies were killed and dissected immediately, to determine their uptake of mff (VmfU) from the skin (Wirtz *et al.*, 1986). The rest of the flies were maintained, at 18–22°C on 10% sucrose solution, for 10 days. All the flies found dead on days 1–9 were immediately dissected and those that survived until day 10 were killed and also dissected, so that the first-, second- and third-stage larvae of *O. volvulus* they contained could be counted, separately (Wirtz *et al.*, 1986).

All of the *O. volvulus* recovered from the flies were preserved, dried on microscope slides (Toe *et al.*, 1997), at room temperature (20–28°C). They were eventually shipped to a specialized laboratory for genetic investigation (the results of which will be reported elsewhere).

Determination of Pharmacokinetic (PK) Parameters

On 'day 1', after admission to the Hohoe hospital, the baseline investigations, and an overnight fast, each subject was treated with ivermectin (as 3-mg tablets donated by Merck & Co., Inc., Whitehouse Station, NJ). All subjects received a single dose of 150 μg/kg. Breakfast was delayed for 2 h after taking the ivermectin tablets. A 10-ml sample of venous blood was collected, in a tube with heparin as anticoagulant, before treatment and 1, 2, 3, 4, 6, 8, 10, 12,

FOI 26-3132 - Document 5

24, 36, 48 and 72 h post-treatment. These samples were centrifuged (2000 × *g*, 15 min) within 30 min of their collection, so that the plasma could be separated off and kept frozen at −70°C until assayed. Plasma concentrations of ivermectin were determined by reversed-phase HPLC (Edwards *et al.*, 1988). The area under the curve of plasma concentration *versus* time to the last experimentally determined concentration (AUC) was determined by the linear trapezoidal rule. Maximum plasma concentrations (C_{max}) and the times at which they were achieved (T_{max}) were noted directly.

Monitoring during Hospitalization and at Follow-up Visits

Adverse events were recorded throughout the admission period and graded as described previously (Awadzi, 1980). Skin snipping and fly feeding were repeated on day 8 for all the SOR, the ivermectin naive and the one subject (known as the 'late convert') who was categorized as an SOR in 1997 and a responder in 2001. All of the subjects were discharged on day 9 but re-admitted on days 90 and 365, for follow-up. At each follow-up visit, the subjects were asked about their general health, whether they had used any drugs or herbal preparations since last seen and, if so, for what purpose. They were given physical and ocular examinations and the skin snipping and fly feeding were repeated on the same groups as at day 8 (except that the 'late convert' was spared fly feeding on day 365). None of the responders (who had been found skin-snip-negative in the field in 1997 and in the hospital in 2001) was subjected to any further skin snipping or fly feeding after the initial examination.

During the day-90 visit, all the skin nodules that could be detected on each subject were excised aseptically, using 2% xylocaine as the local anaesthetic. Each extracted nodule was placed in sterile Eagle's minimum essential medium (MEM) supplemented with 10% foetal calf serum and antibiotics, and examined under a dissecting microscope. Samples of the extruded contents of any damaged nodules were taken, and examined, as wet smears, for embryos. The adult male and female worms in the nodules were harvested by collagenase digestion (Schulz-Key *et al.*, 1977). All the fragmented and calcified worms were noted and then discarded. An inverted microscope was used to check the culture medium around each nodule, and the genital tracts of intact or damaged female worms, for mff. Preferentially, embryogrammes were made from intact female worms and examined, as described by Schulz-Key *et al.* (1980). Embryogrammes were described as 'active' if developmental stages up to mff were present and 'inactive' if the adult females contained empty uteri or one-cell embryos only.

The mff obtained from the skin snips were either stored dry in microtitre plates, after isopropanol treatment, or in 2-ml cryotubes containing 1 ml isopropanol each. Each of the adult male and female worms recovered and each of the deposits from the embryogrammes was fixed in 1 ml isopropanol or 1 ml ethanol, in a 2-ml tube. This preserved material, like that from the fly-feeding experiments, was shipped to a specialized laboratory for genetic investigation.

End-points and Criteria of Parasite Sensitivity to Ivermectin

PRIMARY END-POINTS

The primary end-points used were the sensitivities of the mff and the adult worms. Sensitivity of the mff was measured as the reduction in the skin count between day 0 (pre-treatment) and day 8, supported by the entomological findings. The sensitivity of the adult worms was evaluated from the embryogrammes and the counts of mff in the skin-snips taken on days 90 and 365.

In order to determine the expected response of the mff at various time-points, the skin-snip results from all the blinded studies conducted in Hohoe Hospital between 1991 and 1998 in which single doses of ivermectin of at least 150 μg/kg were administered were

FOI 26-3132 - Document 5

pooled. [Efficacy against the mff and adult worms is not improved by increasing single (Awadzi *et al.*, 1995, 1999) or multiple (Gardon *et al.*, 2002) doses of ivermectin beyond the standard dose of 150–200 μg/kg.] All of the pooled data came from skin-snips taken, in the same way and by the same individual, as in the present study. The skin counts recorded for individual patients on days 8, 90 and 365 post-treatment were categorized, as percentage reductions in the pre-treatment counts, and summarized (Table 2). On the basis of these data, the mff in a subject of the present study were considered sensitive to ivermectin if (1) the level of the microfilaridermia on day 8 showed a reduction of at least 60% compared with the initial level and (2) no blackflies fed on the subject on day 8 contained L_3 when dissected 10 days later.

The prime indicator of a sensitive adult female worm, in SOR or responders, was an inactive embryogramme on day 90 (all worms from the ivermectin-naive were expected to have active embryogrammes on day 90). Additional criteria were day-90 and day-365 counts of mff in the skin that did not exceed 6% and 40% of the day-0 counts, respectively (Table 2). In subjects without nodules and the ivermectin-naive, the sensitivity of the adult worms had to be based entirely on the counts of mff in the skin.

SECONDARY END-POINTS

The secondary end-points were the PK parameters (AUC, C_{max}, and T_{max}), which, owing to inter-subject variability (Awadzi *et al.*, 1994), were described in terms of medians and inter-quartile ranges (IQR). Values of AUC and C_{max} at or below the lower quartile were assumed to indicate reduced bio-availability. For T_{max}, values at or below the lower quartile indicate a

TABLE 2. *Changes in the counts of microfilariae in the skin (pooled data from patients hospitalized in Hohoe, Ghana) with time after first treatment with ivermectin*

Feature	Ivermectin dose (μg/kg) 150	Ivermectin dose (μg/kg) 200–800*	Criterion of adequate parasite response
No. of subjects	35	81	
Initial count (microfilariae/4 mg skin)†	64.2–979.0	52.3–557.9	
NO. OF SUBJECTS WHO, ON DAY 8 POST-TREATMENT, SHOWED:			
<60% reduction in count, compared with initial count	0	0	
60%–80% reduction in count, compared with initial count	3	1	
>80% reduction in count, compared with initial count	32	80	
			⩾60% reduction
NO. OF SUBJECTS WHO HAD A DAY-90 COUNT THAT WAS:			
<6% of the initial count	34	81	
6%–10% of the initial count	0	0	
>10% of the initial count	1	0	
			⩽6% of initial
NO. OF SUBJECTS WHO HAD A DAY-365 COUNT THAT WAS:			
<20% of the initial count	27	75	
20%–40% of the initial count	6	6	
>40% of the initial count	2	0	
>100% of the initial count	0	0	
			⩽40% of initial

*Efficacy was not improved by increasing the single dose beyond 200 μg/kg.
†Counts represent the sum of the densities (in microfilariae/mg) at each of the iliac crests and each of the calves.

EOI 26-3132 - Document 5

relatively rapid appearance of ivermectin in the systemic circulation. These PK parameters were examined in the subjects who showed less-than-expected reductions in their microfilaridermias or had active embryogrammes, to determine if inadequate exposure to ivermectin was a factor in their sub-optimal response. In subjects with normal parasite responses it was presumed that an adequate exposure had occurred.

Data Management

Throughout the study, each subject was considered separately. The results observed were compared with the (previously defined) expected values, so that decisions could be made about the subject and the parasites that he harboured. Pooled data were used only in the determination of cut-off points for the PK parameters. Most of the data are presented in a tabular format and for individual subjects; they are equivalent to case reports. The baseline data are presented for all subjects. The sensitivities of the adult worms, as based on the embryogrammes and skin counts, are summarised for the SOR who had nodules and for the ivermectin-naive. As the subsequent summaries are limited to those subjects whose parasites showed an abnormal response to ivermectin, no post-treatment data are tabulated for any subject (whether a responder, SOR or ivermectin-naive subject) whose parasites responded as expected. Some SOR of special interest are mentioned in the text. The entomological results from this study and the results of the genetic investigation of the parasitic material collected will be presented in detail elsewhere.

RESULTS

The procedures for subject selection and examination, the target criteria for parasite sensitivity and the final reclassification of subjects participating in the trial are summarized in Figures 1 and 2.

The 42 subjects who agreed to participate in the investigation were all males and all completed the hospitalized treatment and follow-up. The essential background features, as assessed at the beginning of the hospital study, are presented in Tables 3 and 4. The SOR from the LBV basin tended to have higher levels of microfilaridermia, more mff in the anterior chamber of the eye and a greater number of nodule sites than the subjects from the Pru river basin (Table 3). Only two responders came from the LBV basin whereas five came from the Pru basin (Table 4). All the ivermectin-naive subjects had nodules and skin counts that were generally higher than those in the SOR, but only three had mff in the anterior chamber of at least one eye (Table 4).

All the participants were in good general health and their haematological and biochemical tests did not reveal any underlying disease. *Mansonella perstans* was found in all the participants from the LBV basin except number 09, in three participants from the Pru basin (numbers 18, 22 and 24) and in subject 32 from the Tordzi basin. Light infections with hookworm (24–792 ova/g faeces) were found in 20 subjects.

Adverse Events

The adverse effects of ivermectin were mild. The most common was bodily pain (eight subjects), followed by headache (seven), then arthralgia, pruritus, gland pain or limb swelling (six cases each), and muscle aches (five). Acetaminophen was given to six subjects for bodily pain, headache, gland pain, joint pain and/or muscle aches, and to two other subjects for low backache unrelated to the treatment. Severe itching of the legs occurred in 18 subjects following fly feeding and was relieved by the local application of calamine lotion.

Parasite Sensitivity to Ivermectin

The skin mff in all but five subjects were found sensitive to ivermectin. The exceptions were subject 07 from the LBV basin (who

FOI 26-3132 - Document 5

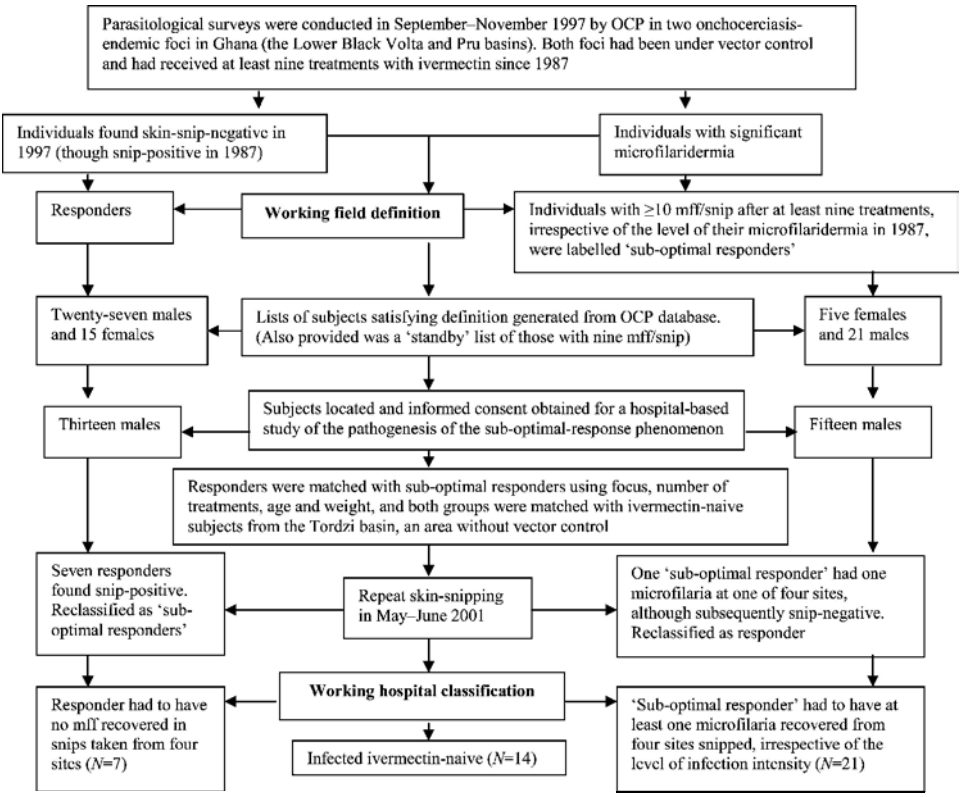


FIG. 1. The approach used to investigate the causes of persistent microfilaridermias despite multiple treatments with ivermectin — subject selection and classification. OCP, Onchocerciasis Control Programme in West Africa; mff, microfilariae.

only showed a 55.4% reduction on day 8, compared with his initial count), subject 16 from the Pru basin (whose skin count increased from 2.0 mff/4 mg on day 0 to 2.4 mff/4 mg on day 8) and subjects 29 and 49 from the Tordzi basin (whose counts increased from 0.6 to 4.7 and 59.3 to 77.4 mff/4 mg, respectively). Infective larvae (L_3) were also found in the blackflies that had fed 10 days earlier (on day 8 post-treatment) on subject 49.

The characteristics of the nodules and the adult female worms, the results of examination of the embryogrammes, and the skin counts on days 90 and 365 (expressed as percentages of the initial counts) are summarized, for the SOR with nodules and the ivermectin-naive, in Table 5. All the embryogrammes of the worms obtained from the nine subjects from the LBV basin (but none of those of the worms from the Pru-basin subjects) were 'active'. The adult worms obtained from six out of seven subjects from the Tordzi basin had active embryogrammes.

The day-90 count of skin mff was below 6% of the initial count in all subjects except numbers 07, 10 and 12 from the LBV basin, number 24 from the Pru basin, and numbers 29, 35 and 36 from the Tordzi basin. By day 365, the adult worms in all but two subjects from the LBV basin (the exceptions being numbers 01 and 06) and those in one subject (number 15) from the Pru basin and one (number 40) from the Tordzi basin were showing a poor response to the drug, with levels of microfilaridermia that were $>40\%$ of those recorded initially. In fact, six out of nine subjects from the LBV basin had more intense microfilaridermias on day 365 than

EOI 26-3132 - Document 5

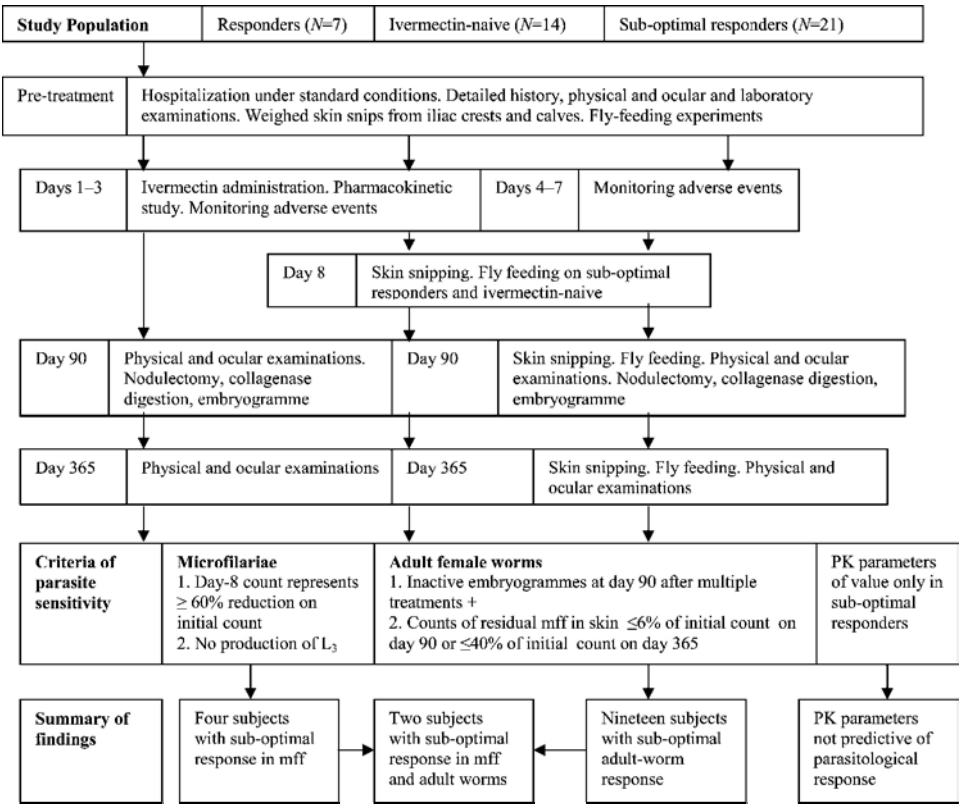


FIG. 2. The approach used to investigate the causes of persistent microfilaridermias despite multiple treatments with ivermectin — the examinations, the criteria for parasite sensitivity, and the main observations made. L_3, Third-stage larvae; PK, pharmacokinetic; mff, microfilariae.

they did when first admitted (data for five of these six subjects are shown in Table 5; the other had no nodules).

Among the SOR without nodules (i.e. numbers 03, 08 and 11 from the LBV basin and numbers 16–20 from the Pru basin), subject 03 was skin-snip-negative on day 90 but had a day-365 count (29.2 mff/4 mg) that was more then four times higher than his initial count (6.2 mff/4 mg). Subject 08 was snip-negative on days 90 and 365 whereas subject 11 was negative on day 90 but had a day-365 count that was 46.7% of his pre-treatment one. Subjects 16, 17, 19 and 20 were snip-negative on days 90 and 365 but subject 18 was snip-positive on both of these days, with counts that were 12% and 136.1% of the initial counts, respectively.

Summary of Parasite Responses to Ivermectin

There were 21 subjects (12 from the LBV basin and nine from the Pru basin) who were classified as SOR at the beginning of the trial. On re-challenge, the results from only six of these SOR — one (number 08) from the LBV basin and five (numbers 17, 19, 20, 21 and 24) from the Pru basin — indicated that their mff and adult worms were sensitive to ivermectin. The 21 subjects with abnormal microfilarial and/or adult-worm responses — two of whom (numbers 07 and 29) had abnormally responsive mff **and** adults — comprised 11 SOR from the LBV basin, four SOR from the Pru basin and six ivermectin-naive subjects from the Tordzi basin (Table 6).

FOI 26-3132 - Document 5

TABLE 3. *Background characteristics of the sub-optimal responders from the Lower Black Volta and Pru basins*

Subject	Village	No. of doses taken*	Age (years)	Weight (kg)	Height (cm)	Onchocercal skin lesions?	No. of nodule sites	Initial microfilaridermia† (microfilariae/4 mg)	No. of microfilariae in anterior chambers of both eyes
FROM LOWER BLACK VOLTA BASIN									
1	Agbelekame I	12	40	60	174.5	No	5	16.1	0
2	Chingakrom	11	48	51.5	164.5	No	1	240.9	1
3	Chingakrom	11	38	61	166.5	No	0	6.2	9
4	Chingakrom	11	48	50.5	162.5	Yes	7	15.4	2
6	Chingakrom	9	43	52.5	173	No	4	55.7	8
7	Agbelekame I	12	57	52	170	No	1	10.1	0
8	Agbelekame I	12	53	60	171	No	0	16.6	0
10	Agbelekame I	12	60	54	168	No	2	10.6	2
11	Agbelekame I	12	51	47	165	No	0	118.2	13
12	Agbelekame I	12	37	59.5	165.5	Yes	2	8.1	0
13	Agbelekame I	12	49	65	176.5	Yes	4	39.8	1
14	Chingakrom	9	38	55	168	No	3	27.9	1
FROM PRU BASIN									
15	Asubende	13	42	67.5	172	No	1	59.1	2
16	Asubende	13	19	70	180.5	No	0	2	0
17	Asubende	13	36	45	163	No	0	0.5	0
18	Asubende	13	51	54	169.5	No	0	19.1	0
19	Asubende	13	18	55	167.5	No	0	14.4	0
20	Asubende	13	21	53.5	169	No	0	5.8	0
21	Asubende	13	24	59.5	171	Yes	1	2.5	0
22	Asubende	13	32	42.5	153	Yes	2	76.7	4
24	Fawomang	13	50	41	159.5	No	2	0.5	0

*Between 1987 and 2000 inclusive.
†Counts represent the sum of the densities (in microfilariae/mg) at each of the iliac crests and each of the calves.

TABLE 4. *Background characteristics of the subjects from the Lower Black Volta and Pru Basins who had shown a good response to ivermectin (the 'responders'), and those of the ivermectin-naive subjects from the Tordzi basin*

Subject	Village	No. of doses taken*	Age (years)	Weight (kg)	Height (cm)	Onchocercal skin lesions?	No. of nodule sites	Initial microfilaridermia† (microfilariae/4 mg)	No. of microfilariae in anterior chambers of both eyes
RESPONDERS FROM THE LOWER BLACK VOLTA AND PRU BASINS‡									
5	Chingakrom	11	48	69	175	Yes	1	0	1
9	Agbelekame I	12	40	53.5	170	No	0	0	0
23	Asubende	13	35	49	161.5	No	1	0	0
25	Fawomang	13	46	47.5	163.5	No	3	0	0
26	Hiampe	13	22	54	164.5	No	1	0	0
27	Hiampe	14	39	63.5	171	No	0	0	0
28	Hiampe	14	35	53.5	170	No	0	0	0
IVERMECTIN-NAIVE SUBJECTS FROM THE TORDZI BASIN									
29	Kpedze-Anoe	0	39	62.5	172.5	No	1	0.6	0
30	Kpedze-Anoe	0	19	50	163.5	No	1	34.3	0
31	Kpedze-Anoe	0	25	62.5	172.5	No	1	26.8	0
32	Kpedze-Anoe	0	21	60	173	No	4	102.9	0
33	Kpedze-Anoe	0	29	48	164	Yes	2	5.6	0
35	Kpedze-Anoe	0	28	55	168.5	No	3	21.5	0
36	Kpedze-Anoe	0	52	47.5	157.5	No	2	111.4	0
37	Kpedze-Anoe	0	41	52	166	No	3	56.5	0
39	Kpedze-Anoe	0	20	53	157.5	No	1	36.9	1
40	Kpedze-Anoe	0	36	55	173	Yes	2	29.2	0
42	Kpedze-Anoe	0	41	64	173.5	No	2	194.6	0
46	Kpedze-Anoe	0	27	60	178.5	No	2	365.1	4
47	Aflakpe	0	31	49	154	No	1	30.2	0
49	Aflakpe	0	43	57	162	No	2	59.3	2

*Between 1987 and 2000 inclusive.
†Counts represent the sum of the densities (in microfilariae/mg) at each of the iliac crests and each of the calves.
‡'Responders' were skin-snip-negative in 1997 and 2001.

FOI 26-3132 - Document 5

TABLE 5. *Embryogrammes and skin microfilarial counts, on days 90 and 365 post-treatment, for the sub-optimal responders (SOR) and ivermectin-naive subjects who were nodule carriers*

		No. of adult female worms on day 90 that were:				Skin microfilariae*				
			'Normal'				Day 90		Day 365	
Subject	No of nodules excised‡	Calcified†	Total	Inactive embryogramme	Active embryogramme§	Initial count	Count	% of initial	Count	% of initial
SOR FROM LOWER BLACK VOLTA BASIN										
1	5	0	5	1	4 (4)	16.1	0	0	3.1	19.3
2	2	0	3	2	1 (1)	240.9	4.1	1.7	114.4	47.5
4	7 (2)	1	8	5	3 (3)	15.4	0.8	5.2	32.3	209.7
6	6	0	18	7	11 (5)	55.7	0.8	1.4	3.1	5.6
7	1	0	3	1	2 (2)	10.1	3	29.7	19	188.1
10	3	2	9	6	3 (1)	10.6	1.2	11.3	15.7	148.1
12	4	2	2	0	2 (2)	8.1	0.6	7.4	13.5	166.7
13	6	1	7	4	3 (2)	39.8	0	0	18.1	45.5
14	1	0	5	2	3 (3)	27.9	0.6	2.2	29.5	105.7
SOR FROM PRU BASIN										
15	1	0	1	1	0	59.1	0	0	38.5	65.1
21	2	1 (1)	0	0	0	2.5	0	0	0.8	32
22	3	3	2	2	0	76.7	18.4	24	7.6	9.9
24	2	2	2	2	0	0.5	0	0	0	0
IVERMECTIN-NAIVE SUBJECTS FROM TORDZI BASIN										
29	1	0	3	1	2 (1)	0.6	0.6	100	0	0
35	3	0	5	4	1 (1)	21.5	1.5	7	5.8	27
36	1	0	2	1	1 (1)	111.4	21.9	19.7	18.1	16.2
40	2	1	8	4	4 (1)	29.2	0	0	11.7	40.1
42	2	0	1	0	1 (1)	194.6	4.7	2.4	37.8	19.4
46	3	0 (1)	28	14	14 (4)	365.1	7	1.9	75.5	20.7
49	2	0	8	8	0	59.3	0.6	1	16.5	27.8

*Counts are given as the number of microfilariae/4 mg skin, and represent the sum of the densities (in microfilariae/mg) at each of the iliac crests and each of the calves.
†The no. of fragmented worms is shown in parentheses.
‡The no. of calcified nodules is shown in parentheses.
§The no. of female worms producing normal microfilariae is shown in parentheses.

FOI 26-3132 - Document 5

TABLE 6. *Summary of results for the subjects who had abnormal microfilarial and/or adult-worm responses to ivermectin*

Subject	Day-8 response of microfilariae			Adult female-worm response			Invermectin pharmacokinetic profile		
	Reduction in count (compared with initial) <60%?	Vector uptake of microfilariae?	Subsequent production of L_3?	Active embryogramme?	Day-90 count of microfilariae in skin >6% of initial?	Day-365 count of microfilariae in skin >40% of initial?	$AUC_{(0-72\,h)}$ (ng/ml.h)	C_{max} (ng/ml)	T_{max} (h)
FROM LOWER BLACK VOLTA BASIN									
1	No	No	No	Yes	No	No	433	19	3
2	No	No	No	Yes	No	Yes	974	47	10
3	No	No	No	(–)	No	Yes	408	22	8
4	No	No	No	Yes	No	Yes	818	36	6
6	No	No	No	Yes	No	No	340	24	3
7	Yes	No	No	Yes	Yes	Yes	313	18	2
10	No	No	No	Yes	Yes	Yes	496	20	6
11	No	No	No	(–)	No	Yes	1103	34	2
12	No	No	No	Yes	Yes	Yes	1245	50	3
13	No	No	No	Yes	No	Yes	1414	45	8
14	No	No	No	Yes	No	Yes	665	21	6
FROM PRU BASIN									
15	No	No	No	No	No	Yes	37	5	6
16	Yes	No	No	(–)	No	No	28	3	2
18	No	No	No	(–)	Yes	Yes	42	3	2
22	No	No	No	No	Yes	No	130	7	3
FROM TORDZI BASIN									
29	Yes	No	No	NA	Yes	No	365	33	6
35	No	No	No	NA	Yes	No	170	9	6
36	No	No	No	NA	Yes	No	239	13	2
39	No	No	No	NA	No	Yes	190	14	3
40	No	No	No	NA	No	Yes	941	54	10
49	Yes	Yes	Yes	NA	No	No	36	9	4

(–), No nodule present; NA, not applicable; AUC, area under the curve of plasma concentration *versus* time; C_{max}, maximum plasma concentration; T_{max}, time to C_{max}.

FOI 26-3132 - Document 5

FOI 26-3132 - Document 5

Pharmacokinetic Parameters

The qualitative assessments of AUC indicated considerable inter-individual variation in the extent of drug exposure. The AUC values obtained, from 40 of the subjects, had a median of 397 ng/ml.h (IQR = 202–856 ng/ml.h). The C_{max} values (obtained from 41 subjects) had a median of 22 ng/ml (IQR = 14–36 ng/ml) and the T_{max} data (obtained from 41 subjects) had a median of 4 h (IQR = 3–6 h). The AUC and C_{max} values for all of the subjects from the LBV basin were within or above the IQR whereas those for all of the subjects from the Pru basin fell below this range. Two of the subjects from the Tordzi basin (numbers 29 and 40) had AUC and C_{max} values within or above the IQR, two (numbers 35 and 49) had AUC and C_{max} values below the range, and two (numbers 36 and 39) had either an AUC value or a C_{max} value that was below this range. The T_{max} values for five subjects from the LBV basin, three from the Pru basin and two from the Tordzi basin were also below the corresponding IQR.

DISCUSSION

Taken together, the present results indicate that the PSM observed despite multiple treatments were most probably the result of the adult female worms failing to respond adequately to ivermectin.

The present evaluation of adult-worm response was based predominantly on the level of reproductive activity of the female worms, as demonstrated in embryogrammes. The embryogrammes of the worms from the LBV were highly active; since the major effect of multiple treatments with ivermectin is the suppression of the production of mff (Chavasse *et al.*, 1992; Duke *et al.*, 1992; Plaisier *et al.*, 1995), this level of reproductive activity shown by the adult female worms was abnormal. In nine out of 11 subjects from the LBV basin, there was a rapid increase in the counts of skin mff between days 90 and 365 following the re-challenge with ivermectin, with day-365 counts that were >40% (in six cases >100%) of the initial values (Table 6). Although the embryogrammes of the worms harvested from all of the SOR from the Pru basin were inactive, three of these SOR had abnormally high counts of skin mff on both days 90 and 365 (one subject) or just day 90 (one subject) or day 365 (one subject); these three SOR presumably harboured adult worms with active embryogrammes but in unknown locations. In six out of seven subjects from the Tordzi basin, embryogrammes were active, as expected following a first dose of ivermectin. Although the counts of skin mff were higher than expected in three of the Tordzi subjects on day 90 and in two others on day 365, they never exceeded the initial count. This contrasts with the findings for the subjects from the LBV basin but is in line with the pooled data summarized in Table 2. The level of reproductive activity of the adult female worms in most SOR from the LBV basin was therefore at least as high, if not higher, than that observed in subjects receiving their first dose of ivermectin.

The mff from all but two of the SOR were sensitive to ivermectin (the exceptions being subject 07 from the LBV basin and 16 from the Pru). The good response to ivermectin of the mff in nearly all of the SOR excludes the possibility that PSM are the result of an incompetent immune system in the host (Ali *et al.*, 2002), and exonerates the mff as important contributors to the phenomenon. Subject 49, who was ivermectin-naive, had a more intense microfilaridermia on day 8 than on day 0, and L_3 developed in the blackflies that fed on him on day 8. Here, the mff fulfilled all the previously defined criteria of a lack of sensitivity to ivermectin. By day 90, however, the level of microfilaridermia in subject 49 had fallen well within expected limits (Table 6). A possible explanation of these observations is that the response to ivermectin of the mff was simply slower than usual, although this in itself implies that the mff were abnormal *ab initio*.

FOI 26-3132 - Document 5

Six subjects originally classified as SOR were found to have ivermectin-sensitive mff and adult worms. This led to a final re-classification in which three subjects from the LBV basin and 10 from the Pru basin were considered responders and 11 subjects from the LBV basin and four from the Pru basin were classed as SOR. The change in the category of six subjects, from SOR to responders, was not the result of a change in the sensitivity of their parasites but reflects the very stringent definition of a 'normal' response employed in the present study. This required that no mff be present in four skin snips taken from areas where the maximum concentrations of mff usually occur (i.e. the iliac crests and calves). Inevitably, some subjects with low numbers of mff in their skin were initially classed as SOR, pending the results of the re-challenge with ivermectin.

The main aim of the ivermectin treatment given to the SOR during the present study was to determine the drug sensitivity of the mff. The sensitivity of the adult worms in the same subjects had already been established by the multiple doses given previously, and was unlikely to be influenced by another single dose. Thus, inactive embryogrammes, coupled with a rapid reduction in the level of microfilaridermia, provide evidence of past and persisting sensitivity of *O. volvulus* adult worms and mff to ivermectin. These were the findings in all six subjects who were reclassified as responders. These six subjects shared some other common characteristics: they appeared to have no (numbers 08, 17, 19 and 20) or very few (numbers 21 and 24) nodules, they had relatively few mff in their skin when admitted (0.12–4.2/mg), they had no mff in the anterior chambers of their eyes, and all except one (number 21) were skin-snip-negative on days 90 and 365 (Tables 3 and 5). These features — which are shared to some extent by the original responders (Table 4), in whom all embryogrammes were found to be inactive — could constitute a working definition of the phenotype of a responder to multiple treatments with ivermectin. No mff were found in any post-challenge skin snips from the 'late convert' (i.e. the original SOR who was re-classified as a responder on admission to the Hohoe Hospital) and none was taken up by any blackfly that fed on him. The same observations would presumably have been made on all the other responders, had they been checked.

The establishment of the exposure of all the participants to at least nine treatments with ivermectin between 1987 and 1997 was essential to the initial definition of a sub-optimal response. Examination of the treatment records provided by the OCP and the Ghana Onchocerciasis Control Unit established that all the eligible inhabitants of the Pru basin had taken 11–14 annual doses by 2000, with the exception of one (subject 24) who had only received nine doses. The data on the treatment rounds in the LBV basin pre-1994 were not as good. The senior author of this article was present, however, at the first treatment round in the LBV basin, and detailed questioning of each of the present subjects from this area (regarding the timing of the doses relative to local and national events) and their descriptions of the adverse effects of each dose over time provided enough evidence to credit each one with the intake of at least nine treatments by 1997. Further treatments were given to the inhabitants of the LBV basin in 1998, 1999 and 2000, with the exception of the subjects from Agbelekame I, who missed the 1998 round.

Detailed personal histories did not reveal any activity, undertaken in the few days before or after an ivermectin treatment, that could have produced an adverse interaction. The results of the clinical and laboratory examinations failed to reveal any underlying disease in any subject. Coincidental infection with *M. perstans* and hookworm were found in several subjects but were unlikely to modify the response of the *O. volvulus* to ivermectin. The PK parameters revealed the expected large inter-individual variation in

FOI 26-3132 - Document 5

circulating plasma concentrations of ivermectin. The main inference from the PK data is that all the LBV subjects had adequate exposure to ivermectin. The significance of the low AUC and C_{max} values in the SOR from the Pru basin is difficult to determine since lower and even undetectable levels of ivermectin (data not shown) were recorded in three of the six subjects (numbers 17, 19 and 24) who were initially classified as SOR but subsequently re-classified as responders. Since the parasites in the latter subjects showed an adequate response to the re-challenge with ivermectin, the PK data recorded in the present study seem to be unreliable predictors of pharmacological response.

The observations listed above make it unlikely that host-related factors were involved in the poor response seen in each SOR. An examination of the 'pill'-related factors also provided no evidence that these played any part in the phenomenon.

The possibility that active embryogrammes and/or high residual numbers of mff in the skin could be the result of new infections was examined in a review of the epidemiological data for the Pru and LBV basins. These data indicated that the residual levels of infection and transmission over the study period were very low. The mean annual transmission potential (ATP), for example, was 45. The ATP is the theoretical total number of infective larvae that would be received in 1 year by an individual stationed at a capture point for 11 h/day and is calculated by adding the corresponding monthly transmission potentials. An ATP of <100 is considered to be a satisfactory result of a blackfly-control programme (WHO, 1995). Thus, in the absence of subject migration into areas of high transmission, new infections were unlikely.

With the elimination of pill and host factors and new infections as the cause of apparent PSM, the only factors that remain relate to parasite tolerance or resistance (Table 2). From the present results, the development of tolerance or the selection for resistance to ivermectin in the adult female worms seems the most likely cause of PSM. It is envisaged that an examination of the genetic profiles of the individual parasites obtained from the various categories of subjects (responders, SOR and the ivermectin-naive) may shed some light on the problem.

In summary, clinical, PK, entomological and parasitological indicators were used to study the responses of individuals and their parasites to ivermectin. Of the 42 subjects from three river basins in Ghana, 21 were found to have *O. volvulus*, predominantly adult female worms, that failed to respond adequately to previous doses or to the first dose of ivermectin; in a small minority of these subjects, the mff also failed to respond adequately. It has been speculated that non-responsive adult worms would have a greater influence on the effective use of ivermectin than non-responsive mff (Grant, 2000). The extent of this phenomenon and any influence it may have had on the control of onchocerciasis in the areas subjected to multiple treatments are unknown. The discovery of sub-optimal response among the ivermectin-naive population supports the concept of a 'first-dose sub-optimal response', possibly related to parasite factors (tolerance). The current results justify the routine monitoring of the efficacy of treatment in any ivermectin-based control programme. Optimally, this should begin when the first pill is given to the first subject.

The results of the present investigation have been discussed informally elsewhere, within the context of current onchocerciasis-control programmes, and more extensive studies have been proposed to define better the sub-optimal-response phenotype and its relationship with ivermectin treatment and any associated changes in parasite genotype.

ACKNOWLEDGEMENTS. The authors thank Merck for supplying the ivermectin tablets. This investigation received financial support from the African Programme for Onchocerciasis Control, the Onchocerciasis Control

FOI 26-3132 - Document 5

Programme in West Africa, and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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FOI 26-3132 - Document 5

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TITLE:

Ivermectin shows clinical benefits in mild to moderate COVID19: A randomised controlled double blind dose response study in Lagos.

ISRCTN40302986

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ABSTRACT

Introduction: In vitro studies have shown the efficacy of Ivermectin (IV) to inhibit the SARS - CoV- 2 viral replication, but questions remained as to In-vivo applications. We set out to explore the efficacy and safety of Ivermectin in persons infected with COVID19.

Methods: We conducted a translational proof of concept (PoC) randomized, double blind placebo controlled, dose response, parallel group study of IV efficacy in RT - PCR proven COVID 19 positive patients. 62 patients were randomized to 3 treatment groups. (A) IV 6mg regime, (B)IV 12 mg regime (given Q84hrs for 2weeks) (C, control) Lopinavir/Ritonavir. All groups plus standard of Care.

Results: The Days to COVID negativity [DTN] was significantly and dose dependently reduced by IV (p = 0.0066). The DTN for Control were, = 9.1+/- 5.2, for A 6.0 +/- 2.9, and for B 4.6 +/-3.2 . 2 Way repeated measures ANOVA of ranked COVID 19 + / - scores at 0, 84, 168, 232 hours showed a significant IV treatment effect (p=0.035) and time effect (p <0.0001). IV also tended to increase SPO2 % compared to controls, p = 0.073, 95% CI - 0.39 to 2.59 and increased platelet count compared to C (p = 0.037) 95%CI 5.55 - 162.55 × 10^3/ml. The platelet count increase was inversely correlated to DTN (r = -0.52, p = 0.005). No SAE was reported.

Conclusions: 12 mg IV regime may have superior efficacy. IV should be considered for use in clinical management of SARS-Cov-2, and may find applications in community prophylaxis in high-risk areas.

Keywords: Ivermectin, COVID-19, RCT, Efficacy, Safety, Days-to-Negative.

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INTRODUCTION

The Corona Virus Disease 2019 (COVID 19) pandemic caused by the Severe Acute Respiratory Syndrome Corona virus -2 (SARS-CoV-2)[1] led to a World Health Organization declared global pandemic on March 11, 2020[2]. As of December 22, 2020, more than 77.7 million people on all continents had being infected and more than 1.7 million people had died globally[3]. Prolonged morbidity after recovery from acute COVID 19[4] and astronomical hospital costs[5], has left hospitals swamped and health workers exhausted. In addition, the global economy is in a depression[6] with massive job losses, furloughing and social movement restrictions.

There have been concerted attempts to seek preventive and interventional modalities to arrest the spread of the contagion by public health measures such as masking, social distancing, self isolation and hygiene, or more recently by vaccines[7,8]

There are also pharmaceutical/ therapeutic agents with antiviral properties, being repurposed to urgently treat or serve as chemoprevention for COVID 19. One such drug is Ivermectin, which has exhibited broad spectrum anti-parasitic, anti-bacterial and antiviral properties against many RNA viruses[9,10]. Ivermectin is extensively used, with good safety profile in Nigeria and other African nations in treating ocular onchocerciasis[11]. Of more current import, Ivermectin was shown to exhibit a 5000-fold reduction in SARS-C0V-2 viral RNA in vitro in Vero-h/SLAM cells in a study from Australia.[12]

There are several mechanisms by which Ivermectin may inhibit SARS-CoV-2 in COVID 19 patients, including by inhibition of RNA -dependent RNA polymerase (RdRP) required for viral replication,[13] abolition of importin-α/β1 heterodimer nuclear transport of SARS-CoV-2 from the cytosol to the nucleus, and inhibition of viral mRNA and viral protein translation[14] .

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However, there has been skepticism as to whether the virucidal IC50 of Ivermectin against SARS-CoV-2 of 2.4uM (obtained in vitro) could be feasible or attainable in humans or patients with COVID 19. This is because a 10-fold dose of Ivermectin (120mg) simulations-based kinetics in cattle still did not yield peak drug levels (Cmax) approaching the IC50 for in vitro SARS-CoV-2 inhibition[15]. In yet another simulation, based on human pharmacokinetics of different potential antiviral SARS-CoV-2 repurposed drugs, Ivermectin was one of the drugs predicted to have 10-fold concentrations higher than their reported 50% effective concentration [EC50].[16] There is thus conflicting report on simulations from cattle and human pharmacokinetics in the effective anti-SARS-CoV-2 concentration attainable by Ivermectin dosing. Ivermectin has a long half life ($t^{1/2}$) of 81-91 hours, and is highly lipophilic with a high volume of distribution (Vd), indicating preferential lung and tissue accumulation.[17]

Pharmacodynamically, Ivermectin dose-dependently inhibits lipopolysacharide (LPS) induced release of inflammatory cytokines (interleukins) in mice and improved LPS-induced survival.[18] Collectively, there are were multiple pharmacodynamic and pharmacokinetic basis that suggest a potential utility and efficacy of Ivermectin in COVID 19.

We therefore tested the hypothesis that Ivermectin will exert a clinically and therapeutically beneficial effect in mild to moderate COVID 19 patients in a randomized double blind controlled clinical trial in Nigerian COVID 19 patients with RT- PCR proven SARS-CoV-2 positivity.

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METHODOLOGY.

The study was a proof of concept (PoC), double blind, randomized controlled trial, of a parallel group, dose-response design. There were 3 treatment groups to which COVID 19 positive patients were randomized. One group received Ivermectin 6mg (given every 84 hours) twice a week, another group received Ivermectin 12mg (given every 84 hours) for 2 weeks, and the third group received lopinavir / ritonavir daily for 2 weeks.

The study protocol received ethical review and approval of the Institutional Review Board of the Lagos University Teaching Hospital (LUTH), Lagos, Nigeria. The protocol was also reviewed and approved by the Nigerian Agency for Food and Drug Administration and Control (NAFDAC) in Lagos.

The patient inclusion criteria were COVID 19 PCR proven positive patients, who gave informed, written consent to participate in the study, and were either asymptomatic or had mild/moderate symptoms.

Study exclusion criteria were COVID 19 negative patients, patients who had COVID pneumonia or requiring ventilator therapy, renal failure, thromboembolic complications, or unconscious by reduced Glasgow Coma Scale.

There were 62 patients randomized to the 3 treatments. COVID 19 PCR testing was undertaken at baseline (pretreatment time 0 hour) and after dosing at 0 hours, 84 hours, 168 hours, 232 hours and 336 hours.

The baseline demographic, clinical, laboratory and virological data of the 3 patient groups is summarized on Table 1.

RT - PCR

COVID 19 test was by RT polymerase chain reaction (PCR) testing for 3 genes. Using a geneexpert machine and testing simultaneously for three genes

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(Orf, EN, N) A positive COVID 19 test requires all the 3 genes to be present, and a negative test requires all the genes to be functionally absent.

The Cycle threshold (Ct) of the gene tester of more than 40 was regarded as negative, and values below 40 are positive where the Ct value bears an inverse logarithmic relationship to the SARS -CoV-2 viral load.[19,20]

Routine biochemistry, hematology, arterial oxygen saturation (Pa02) temperature and clinical data were gathered, and prognostic ones were recorded at the aforementioned times.

RESULTS.

This study was undertaken between May and November 2020. A general description of the study population and the spread over three arms is found in Table 1. Sixty-three patients with positive PCR result were randomised into three arms of the study. There was one withdrawal, thus sixty-two patients completed the study. The average age was 44.1years (SD14.7), ranging from 20-82. There were 43 males and 19 females. The patients had mild to moderate clinical symptoms and none of them required ventilator, although five required intranasal oxygen, 3 in the 12mg arm (B) and two in the control arm (C). One third of the patients reported with a fever and cough, while 44% and 18% respectively reported with headache and difficulties with breathing. 12% reported with anosmia/ageusia. The commonest comorbidities were Diabetes Mellitus (DM) (2) and Hypertension (9), while some had combined hypertension and DM. Some patients required concomitant medications such as dexamethasone, enoxaparin, and supplemental oxygen.

The effectiveness of randomization was assessed, and the results are displayed in Table 1. In all, twenty-one patients were each randomised into the 6mg (A) and 12mg (B) Ivermectin arms while twenty went into the control arm (C).

There was no significant difference in the distribution of the age, sex and symptoms, comorbidities, blood counts, prothrombin time, liver function and

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kidney function tests. There were however slight differences in the baseline Cycle threshold (Ct) values, being lower in the A arm than the other two arms with regards to the ORF and N genes, but similar for the EN gene. The distribution of other supplemental medications taken by participants, aside from Ivermectin, was broadly similar. These included Zinc, ascorbic acid, vitamin D and Azithromycin.

Changes in variables over time was assessed.

The time to SARS-CoV-2 negativity is described in Figures 1A and B and Table 2. Ivermectin significantly and dose-dependently reduced time to negativity compared to the control group. The mean days-to-negative in the control arm was 9.15 (CI 5.68 to 12.62) while for combined Ivermectin arms it was 5.33 (CI 4.33 to 6.32). Average days-to-negative in the combined Ivermectin arms (any Ivermectin) was thus shorter by 3.83 days (95% CI 6.54 to 1.11) compared to controls P=0.0066. (Student t-test.)

Mean days-to-negative for the 12mg arm was however shortened by 4.5 days, and by 3.15 days for the 6mg arm compared to controls. These differences were significant by ANOVA P>F =0.0179.

The distribution of the days-to-negative are depicted in Figure 1A. Figure 1B depicts a Kaplan Meier (KM) curve comparing time to negative (failure) for the three arms. Log rank test for equality of survival functions gave P>chi = 0.0363. Figure 1C depicts KM curve comparing 'Any Ivermectin' with controls. P>chi=0.019.

Cox proportional hazard model enabled computation of Hazard Ratio (HR) using the control arm as base. HR for the 6mg arm was 1.68, (P=0.120, CI 0.87 - 3.25) while for the 12mg arm, HR was 2.38 (P=0.011, CI 1.22 - 4.65). This suggests that the 12 mg treatment arm will cause the patient to progress 2.38 times faster than the control group. Although there is a HR of 1.68 for the 6mg arm, it did not achieve statistical significance.

The HR for "Any Ivermectin" was 1.96, P= 0.024, CI 1.09 -3.51.

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A 2-way 2 factor Repeated Measure Analysis of Variance (RAMOVA) concerning SARS-CoV-2 PCR assays was carried out to compare means and variances of each of the three treatments at the different time points to obtain treatment effect, Time effect and Time Treatment interaction. A plot of the Means and Standard Error of the Means of 20 patients in each of group A, B, C is shown in Figure 2. There was a significant treatment ($p=0.035$) and time effect ($p < 0.0001$) of Ivermectin on COVID 19 ranked scores compared to controls.

The likelihood of negativity by day five was explored. The Ivermectin arm was 3.45 times more likely to go negative by or before day five, P=0.0271, 95% CI 1.12-10.63. This effect is slightly mitigated by sex (adjusted OR 3.44 P>z 0.031 CI= 1.12 -10.6) but more significantly by age (OR 2.77 P>z 0.113 CI= 0.79 to 9.8).

Changes in clinical and laboratory parameters at baseline and at seven days (or as otherwise stated) were observed for the three arms and recorded in Table 3. Day seven was used as a midway point in the trial. Of note was that there was a moderate increase in SpO2 in the Ivermectin arm, although this did not attain significance (P=0.098). The difference from baseline to highest attained SpO2 during the study for each participant is also depicted in Figure 3A, in which baseline figures are compared with highest attained SpO2. (P=0.073)

Because the drug Ivermectin is essentially metabolized in the liver via CYP34A, changes in liver enzymes such as SGOT (serum glutamic-oxaloacetic transaminase,) SGPT (Serum glutamic pyruvic transaminase) and Alkaline Phosphatase were analysed. Both arms of treatment showed reduction in SGOT and SGPT and moderate increases in Alkaline phosphatase. None of these were statistically significant.

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There were also no significant differences in changes in kidney function tests such as Blood Urea Nitrogen and Creatinine between the three groups.

There was a notable significant increase in platelet counts in the ivermectin arm relative to the control arm (P=0.037). See Figure 3B. There was also a moderate but not significant relative increase in lymphocyte count. The overall Platelet Lymphocyte Ratio (PLR) was not significantly changed (Table 2). In Figure 3C, we note a statistically significant (P=0.0055) negative correlation between days-to-negative and increase in platelet count. The higher the change in platelet count, the fewer the days-to-negative. Pearson's r= -0.53, R^2=0.28

There were slight increases in prothrombin time across all arms, more so in the control arm. Rate of increase was not significantly different across arms.

There were no significant changes over time, across the three groups in clinical parameters such as Respiratory rate, Heart rate, Temperature, and symptoms such as cough and dyspnea as assessed by Likert scales.

There was no significant overall effect of age on days-to-negative by linear regression analysis (r =0.046 p = 0.728). However, those in the 30-40-year age band had a lower time to negative relative to others.

No adverse effects of Ivermectin was reported in response to questioning or spontaneous report.

Symptomatic improvement was seen in all patients, with resolution of fever dypnea and other signs. There was no mortality and the patients remained well on follow up.

DISCUSSION

Our findings show a statistically significant and dose dependent effect of Ivermectin to reduce the time to SARS-CoV-2 negativity in RT-PCR COVID 19 positive patients (p =0.0066) and significant treatment (p=0.035) and

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time effect (p <0.0001) of Ivermectin on COVID 19 ranked scores compared to controls by RAMOVA. A Hazard Ratio of 2.38 in the 12mg arm suggests that negativity events such as described above are likely to occur more than twice as fast with Ivermectin. In addition, Ivermectin appears to be 3.45 times more likely (P=0.0271, 95% CI 1.12-10.63.) to induce negativity by day 5 compared to controls. Collectively, these results demonstrate a likely beneficial treatment effect of Ivermectin, to reduce the duration of illness, elicit faster recovery and diminution of qualitative indices of SARS-CoV-2 viral load compared to the usual treatment.

These results are consistent with those of Ahmed et al[21] who demonstrated a significant reduction in time to COVID 19 virological clearance with Ivermectin of 3 days on average, albeit at a higher dose of 12mg daily for 5 days in Bangladesh. Similar findings were reported by Elgazzar and colleagues in Egypt[22].

Our results demonstrate a dose-response in the effects of Ivermectin 6mg and 12mg twice weekly in time to SARS-CoV-2 negativity. The time (mean +/- SD) to negativity (days) in the treatments were; 6mg; 6+/-2.95 , 12 mg; 4.65 +/- 3.19 , and the controls 9.15+/- 7.26, p =0.02 ANOVA. When both Ivermectin groups were combined (with a new mean 5.34 +/- 0.07 days n=41) the mean difference SEM from placebo control, of - 3.81 +/- 1.34 days was statistically significant p = 0.0066, 95% CI -1.13 to - 6.49 days.

These collectively suggest that the 12 mg dose achieved a faster SARS-CoV-2 virological clearance compared to the 6 mg dose, and that using these standard doses of Ivermectin employed in onchocerciasis treatment caused a 3.8 days faster virological clearance, and clinical amelioration compared to controls on lopinavir/ritonavir.

The findings also provide Proof of concept (Poc) of Ivermectin efficacy against SARS-CoV-2 at the doses that were initially considered inadequate based on pharmacokinetic simulations[15,17]

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The progressive reduction in COVID 19 positivity in the Ivermectin-treated group over time, as well as the treatment effect were sustained with no time-treatment interactions (Figure 2. Table 2). This was associated both with symptomatic improvements as well as Ivermectin-induced reversal of abnormal COVID 19 prognostic parameters. Ivermectin treatment was associated with a strong trend to an increase in arterial oxygen saturation (SPO2%) compared to controls (See Figure 3A) p= 0,073 and 95% CI of -0.39 to 2.59. Pulse oximetry at the finger digits, has recently been shown to exhibit racial divergences with a higher likelihood of overrating SPO2% and leading to "occult hypoxemia" occurring more in black people in comparison to whites[23] thus, the real change in SPO2 with Ivermectin may have been masked.

Platelet count is another prognostic index in COVID 19 with thrombocytopenia reflecting platelet consumption in SARS-CoV-2 as part of sepsis-induced coagulopathy, even before frank Disseminated Intravascular Coagulation (DIC) manifests as hemorrhage or thrombosis[24]. Ivermectin treatments (combined 6mg and 12 mg dose) caused an increase in platelet count relative to the control group patients with a 95% CI for the difference of 5.55 to 162.55 x 10^9/L p= 0.037 ANOVA, F =4,88, df -24. (See Table 3). Further, there was an inverse correlation (r = - 0. 52 , R^2 = 0. 28, p = 0.005) between the Ivermectin induced increase in platelets and the time to SARS-CoV-2 negativity in the patients (See Figure 3C). These platelet results indicate an Ivermectin effect to reverse a negative prognostic factor, and the increase in platelets is associated with a faster resolution and inhibition of SARS-CoV-2 virological proliferation.

The baseline prothrombin time appeared slightly prolonged in all the patients (>0.15 seconds), but no effect of treatment in any group was apparent. The concurrent trend to Ivermectin improvement of COVID 19- associated arterial

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hypoxemia, concomitant with a reduction in prothrombotic hematological coagulopathy (reversal of platelet consumption) may be a consequence of ivermectin anti-inflammatory reduction in COVID-19 hypercytokinemia ("cytokine storm'), and is entirely consistent with reduction in mortality and especially in patients with SARS-CoV-2 pulmonary complications.[25-28] Meta analysis revealed that IL-6 and associated cytokines are correlated with SARS-CoV-2 severity in patients[29] which are under Janus Kinase (JAK-STAT) signaling pathway.[30] Given the known effect of Ivermectin to inhibit IL-6, TNF-alpha in vivo and in vitro, and its suppression of NF-kappa B translocation in mice,[18] a formal controlled study of the effect of Ivermectin on hyper-cytokinemia is now indicated in COVID 19.

There were no significant changes in hepatic and renal functions given that Ivermectin is hepatically metabolized. The gender of participants did not exert any effects on the pharmacodynamic effects of Ivermectin in time to COVID 19 negativity. However, the likelihood of negativity by day 5 was mitigated by age from 3.45 to 2.77.

Ivermectin was remarkably well tolerated and there was no adverse drug event reported spontaneously or in response to inquiry.

In conclusion, Ivermectin exhibited a dose-dependent significant inhibitory effect on SARS-CoV-2. This study provides support for the translation of the in vitro findings of Caly et al[12] at doses that were initially thought to be suboptimal in humans. The 12mg twice weekly regime appears to confer a superior efficacy. Ivermectin modified prognostic factors such as arterial oxygenation and platelet and hematological indices of SARS-CoV-2 infections.

The mechanisms of the benefits were not established in this study, but an effect on JAK-STAT and NF-kappa B related cytokines should be studied in patients.

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Further study of Ivermectin 12mg in the pre-exposure prophylaxis and prevention of community transmission of SARS-CoV-2, such as spoke-and-wheel or targeted hotspots treatment, is now warranted, especially as an interim measure in countries that cannot immediately roll out vaccination programs.

LEGENDS TO FIGURES

Figure 1A. Boxplot showing distribution of Days to Negative between the three arms of the study: A=6mg Ivermectin, B=12mg Ivermectin C=Control

Figure 1B. Kaplan Meir Curve showing time sequence of days to negativity in the three arms of the study.

Figure 1C. Kaplan Meir Curve showing time sequence of days-to-negativity, 'Any Ivermectin' versus Control.

Figure 2. **2-Way Repeat Measures of Analysis of Variance (RAMOVA) SARS CoV-2 PCR ASSAY.**

Figure 3A. Difference in change in SpO2 from baseline to highest attained in study.

Figure 3B Change in platelet count (Day7-Baseline) Ivermectin group versus control (Standard of Care)

Figure 3C Scatterplot exploring relationship between change in platelet count and Days- to-negative PCR. r= -0.53, R^2=0.28 P>F=0.0055.

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Table 1: Distribution of baseline Variables by Trial arm (SD/95%CI)

Variable	Trial Arm			Overall	P value
	6mg Ivermectin	12mg Ivermectin B	Control C		
Number per arm	21	21	20	62	
Age (years)	48.3	39.7	44.8	44.1(14.7)	0.180
Male	15	14	14	43	0.924
Female	6	7	6	19	
Symptoms					
Fever	30%	42.8%	20%	31.2%	0.28
Headache	50%	57%	25%	44.3%	0.096
Cough	30%	19%	45%	31.5%	0.198
Dyspnea	20%	23%	10%	18.0%	0.497
Signs					
Anosmia/Ageusia	25%	15%	0	12.7%	0.081
Resp. rate /min	26(6.2)	23.6(6.9)	23.7(6.3)	24.2(6.4)	0.7
Temp C^o	36.4	36.3	36.6	36.4	0.29
Comorbidities					
Hypertension	2	2	5	9	
Diabetes M	1	1	0	2	
Labs					
Platelet count/ml (000)	215(68)	241(128)	211(69)	223(93)	0.57
Prothrombin time					
Lymphocyte count/ml (000)	2.0	1.8	2.1	1.9	0.70
SpO2	97.5(1	96.8(2	95.8(6.4	96.7(4.2)	0.64

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	.4)	.2)	)		
Prothrombin time	18.2(4.5)	16.3(2.5)	18.1(4.8)	17.5(4.0)	0.36
SGOT units/l	55.7(36.1)	45.0(18.8)	41.3	47.5(25.2)	0.37
SGPT units/l	55.6(52.5)	32.5(24.3)	24.6(14.7)	36.2(34.1)	0.08
Alkaline phosphatase units/ml	65.4 (24.5)	65.9(26.2)	58.8(17.6)	63.3(22.5)	0.68
Blood Urea Nitrogen BUN mg/dl	29.23(7.9)	24.05(7.9)	28.38(4.5)	27.23(7.0)	0.29
Creatinine mg/dl	0.87 (0.21)	0.84(0.18)	0.88(0.27)	0.86(0.21)	0.930
Cycle Threshold (Ct) (ORF gene)	27.8(7.3)	33.12(5.0)	33.1(5.1)	31.2(6.3)	0.049
Ct EN gene	23.9(7.3)	26.6(17.1)	21.4(13.1)	24(11.5)	0.83
Ct N gene	21.5(9.1)	28.2(12.6)	32.1(3.9)	27.9(9.2)	0.02
Medication					
Dexamethasone	1	1	2	Not additive	Similar
Zinc	16	17	18	Not Additive	Similar
Supplemental oxygen use	0	3	2	Not additive	Similar
Enoxaparine	2	1	1	4	Similar
Total Bilirubin mg/dl	1.0	0.7	1.3	1.0	0.12

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Table 2: Days to negative in the three trial arms.

Trial Arm	Mean days-to- negative (95% Confidence Interval)	Standard deviation	N
A 6mg Ivermectin	6.0 (4.61 - 7.38)	2.96	20
B 12mg Ivermectin	4.65 (CI 3.15- 6.15)	3.2	20
A&B (Any Ivermectin)	5.33 (CI 4.33-6.32)	3.12	40
C Control	9.15 (CI 5.68- 12.62)	7.42	20
Total study population	6.6 (CI 5.25-7.95)	5.24	60

Tests were not completed in two cases.
The one-way ANOVA P>F= 0.017.
T-test comparing “Any Ivermectin” and control P=0.0066

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Table 3: Changes in variables over time, Ivermectin versus Control arm

Variable	Change (Day 7-Baseline) (unless otherwise stated.)			95% CI	P value (Period)	Interpretation
	Ivermectin	Control	Mean difference control-Ivermectin			
Liver enzymes						
SGOT units/l	-4.9	-6.0	1.06	-17.0-19.1	0.89	Both Ivermectin and control arms show reduced SGOT
SGPT units/l	-6.75	-4.95	-1.76	-32.06 -28.46	0.89	Ditto
Alkaline Phosphatase units/ml	8.96	4.71	4.24	-39.4 - 48.24	0.84	Moderate increases in Alkaline Phosphatase both arms
Kidney functions						
BUN mg/dl	3.82	-9.56	13.38	-17.33 - 44.11	0.32	Slight increase in the Ivermectin arm
Creatinine mg/dl	.058	0.006	0.052	-0.21 - 0.32	0.881	Slight increase both arms
Hematology						

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Platelet count (000/ml)	20.05	-64.00	84.06	5.56 - 162.55	0.0369*	Significantly higher increase Ivermectin arm
Lymphocyte (000/ml)	0.269	0.163	0.106	-0.71 - 0.92	0.792	Slightly higher increase Ivermectin arm
PLR Ratio	-31.65	23.70	-55.35	-148.19 - 37.49	0.2299	No significant change
SpO2 %	0.125	-1.444	1.56	-0.85 - 3.99	0.0975* (Day 2-1)	Slightly higher increase in PaO2 Ivermectin arm
Prothrombin time (Secs)	0.97	1.94	-0.96	-4.91-2.99	0.61	Prothrombin time increases more in control arm
Clinical Parameters						
Respiratory Rate/min	-1.27	-4.0	2.72	-3.93 - 9.39	0.390	No difference
Heart Rate/min	-2.52	-0.18	-2.33	-9.35 - 4.68	0.505* (Day 3-2)	No difference
Heart Rate/min	-12.83	-6.0	-6.83	-31.29 - 17.62	0.556 Day 7-Baseline	No difference
Symptoms						
Cough **(Likert scale)**	-0.06	-0.2	0.15	-0.15 - 0.44	0.310	No difference

FOI 26-3132 - Document 5

Temperature C^{o}	0.08	0.37	-0.29	-0.79 - 0.21	0.244 (Day3-2)	No difference
Dyspnea (Likert)	0	-0.2	0.2	-0.18 - 0.58	0.29	No difference

SGOT: Serum glutamic-oxaloacetic transaminase
SGPT: Serum glutamic pyruvic transaminase

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LEGENDS TO FIGURES

Figure 1A. Boxplot showing distribution of Days to Negative between the three arms of the study: A=6mg Ivermectin, B=12mg Ivermectin C=Control

Figure 1B. Kaplan Meir Curve showing time sequence of days to negativity in the three arms of the study.

Figure 1C. Kaplan Meir Curve showing time sequence of days-to-negativity, 'Any Ivermectin' versus Control.

Figure 2. **2-Way Repeat Measures of Analysis of Variance (RAMOVA) SARS CoV-2 PCR ASSAY.**

Figure 3A. Difference in change in SpO2 from baseline to highest attained in study.

Figure 3B Change in platelet count (Day7-Baseline) Ivermectin group versus control (Standard of Care)

Figure 3C Scatterplot exploring relationship between change in platelet count and Days- to-negative PCR. r= -0.53, R^2=0.28 P>F=0.0055.

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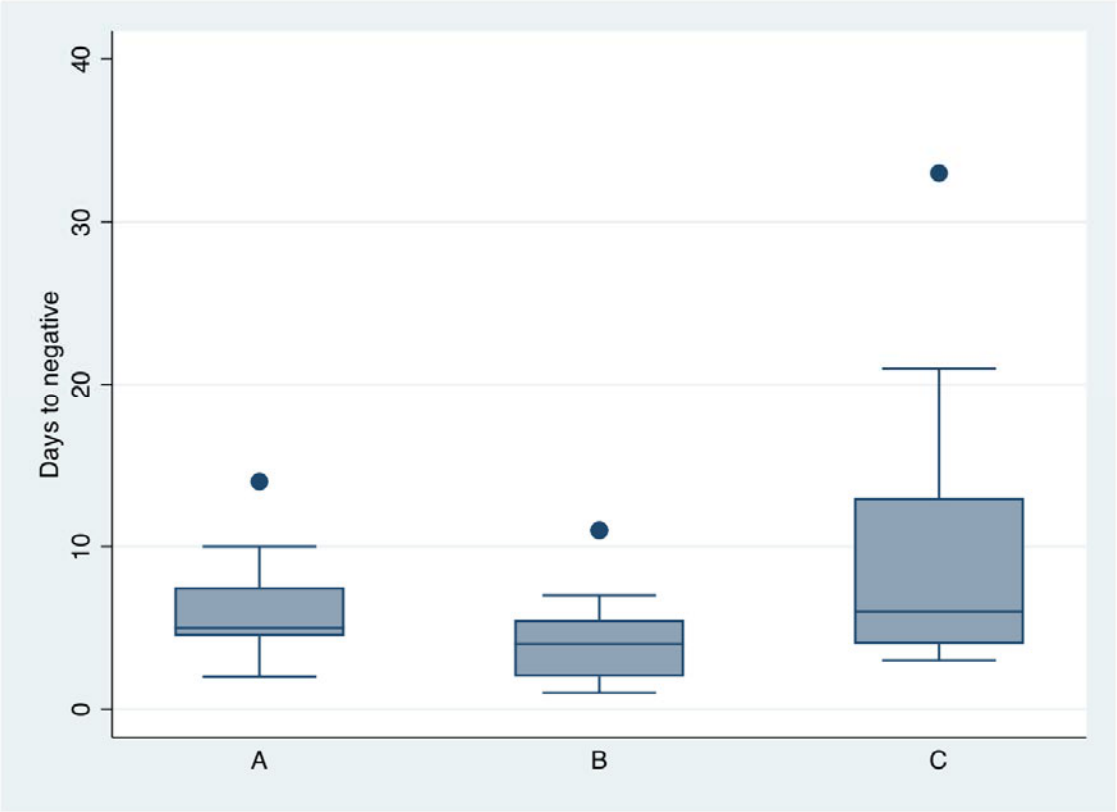


Figure 1A: Boxplot showing distribution of Days to Negative between the three arms of the study: A=6mg Ivermectin, B=12mg Ivermectin C=Control

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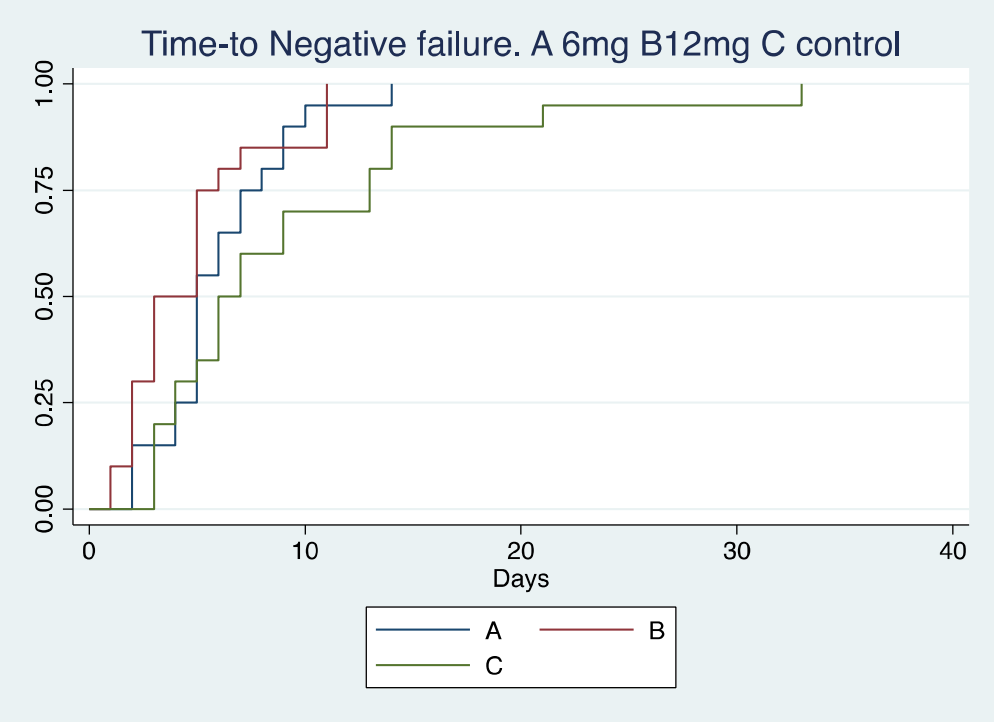


Figure 1B: Kaplan Meir Curve showing time sequence of days to negativity in the three arms of the study.

Log Rank P>Chi= 0.036.
Hazard Ratio for 6mg arm 1.68, (P=0.120, CI 0.87 to 3.25)
Hazard Ratio for 12mg arm 2.38 (P=0.011, CI 1.22 to 4.65)

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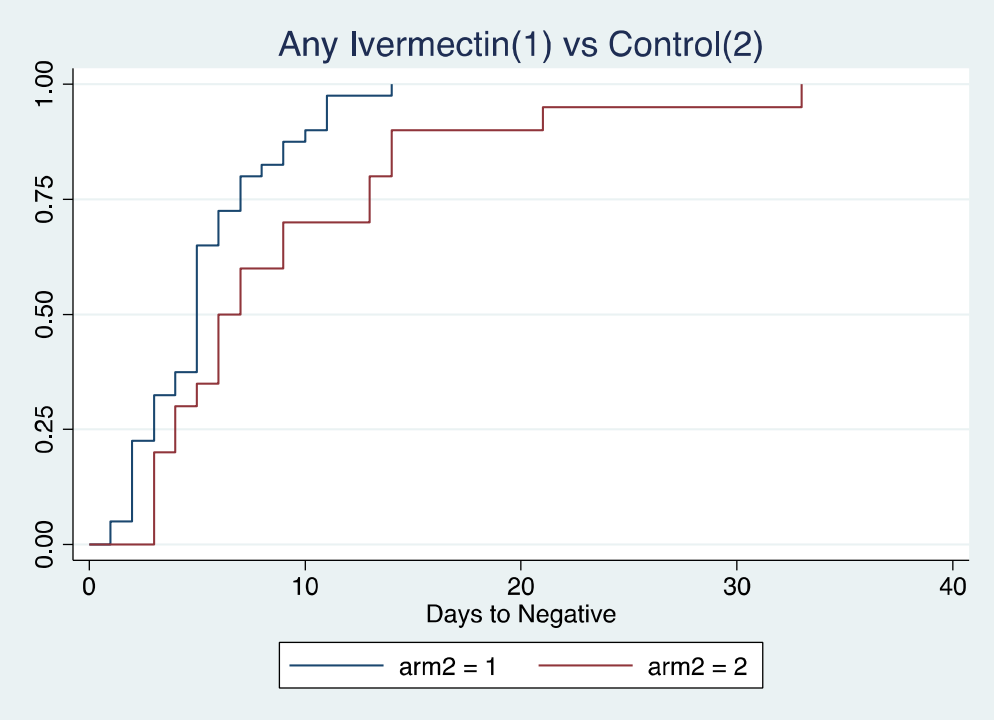


Figure 1C: Kaplan Meir Curve showing time sequence of days-to-negativity, 'Any Ivermectin' versus Control.

Log Rank P>Chi= 0.019.
Hazard Ratio 1.96, P= 0.024 CI 1.09 -3.51

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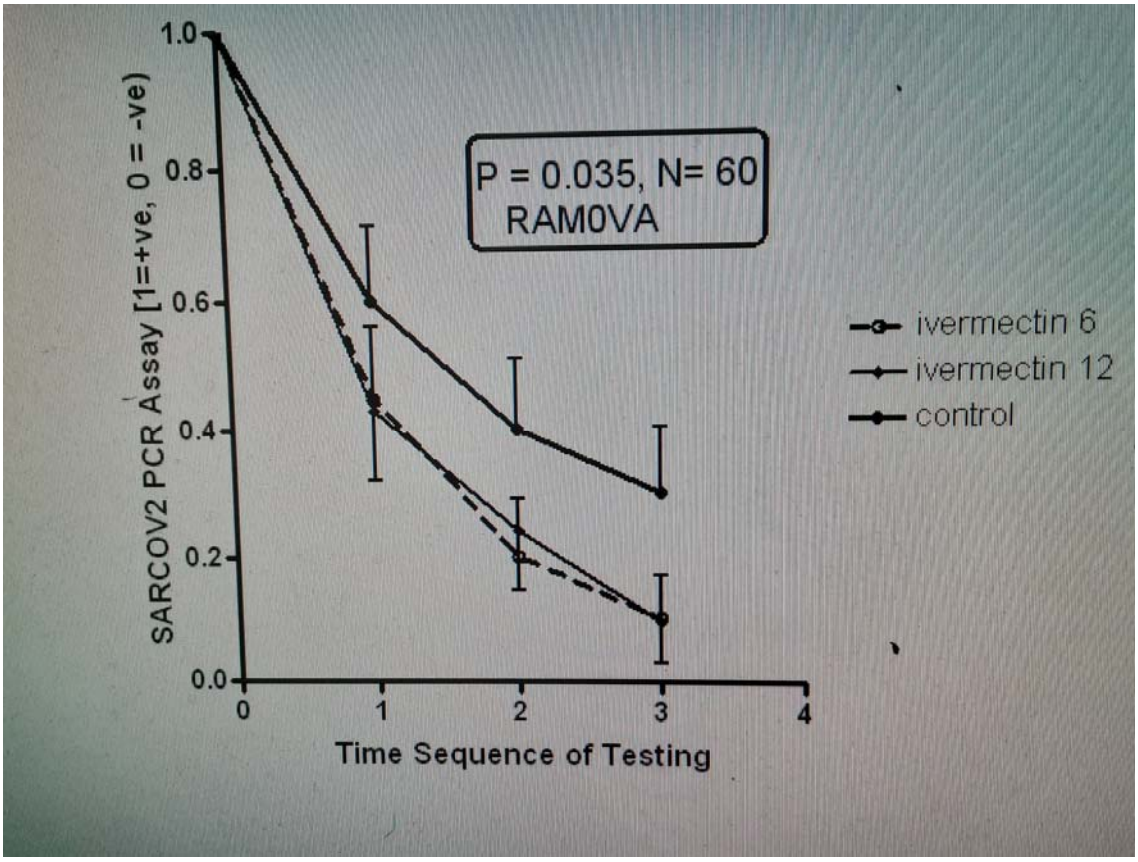


Figure 2: **2 -Way Repeat Measures of Analysis of Variance (RAMOVA) SARSCOV2 PCR ASSAY.**

Key: Time sequence 1=+84hrs 2=+168hrs 3=+232hrs 4=+336hrs

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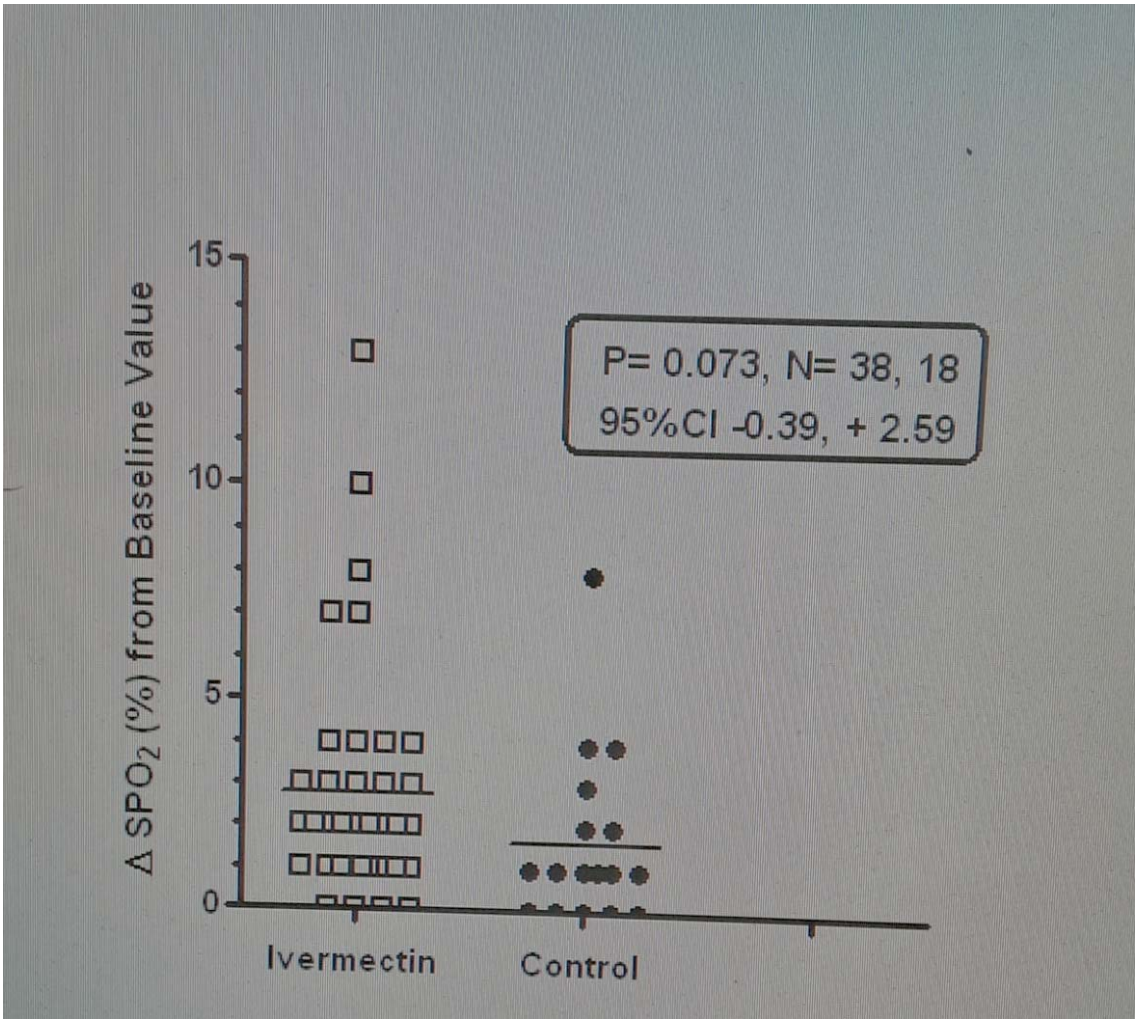


Figure 3A: Difference in change in SpO2 from baseline to highest attained in study.

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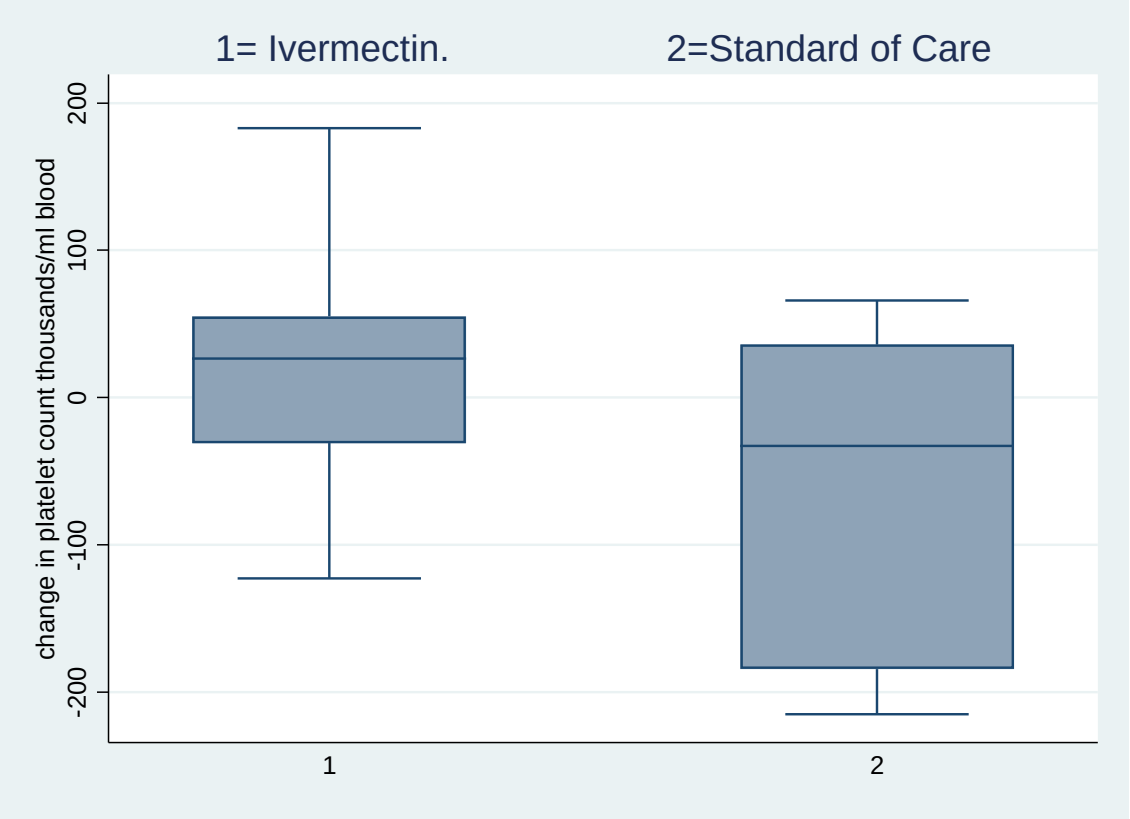


Figure 3B: Change in platelet count (Day7-Baseline) Ivermectin group versus control (Standard of Care)

Mean difference = 84.05556 95% CI =5.56 - 162.55, P= 0.0369.

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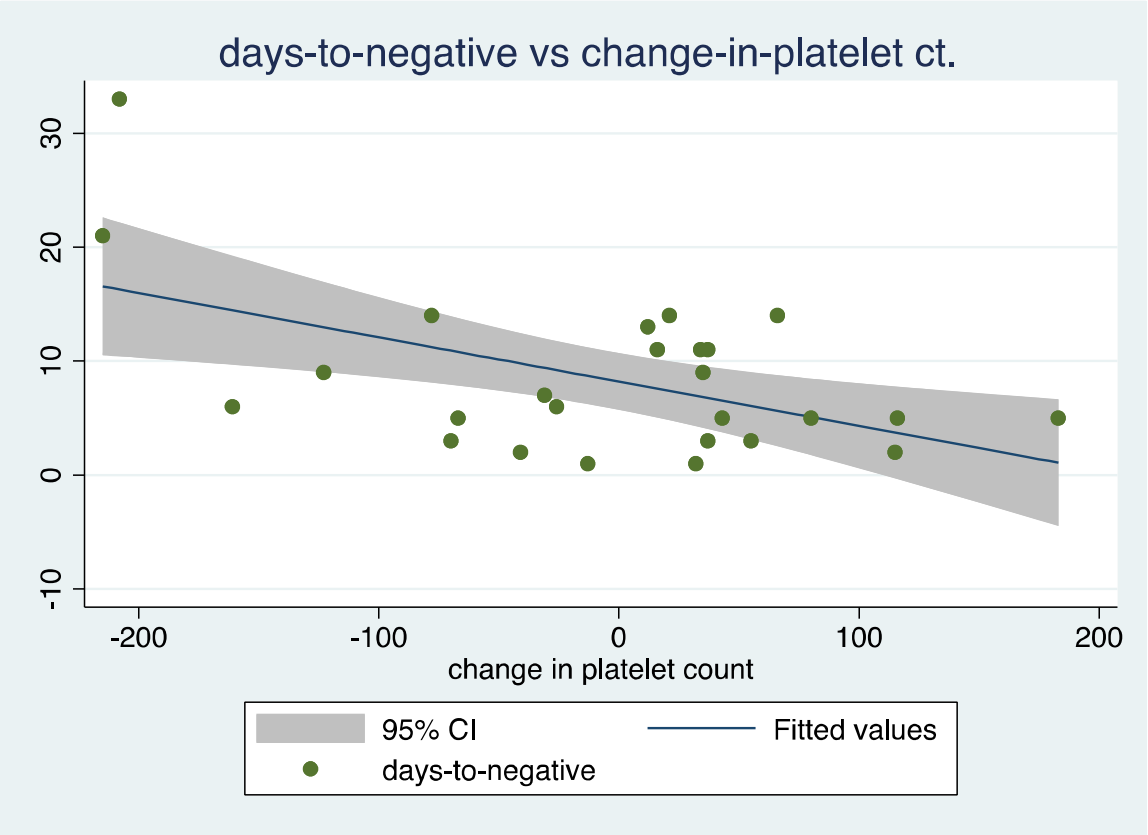


Figure 3C: Scatterplot exploring relationship between change in platelet count and Days- to-negative.
PCR. r= -0.53, R^2=0.28 P>F=0.0055.

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FOL26-3132 - Document 5

REVIEW ARTICLE

https://doi.org/10.1038/s41564-021-00958-0

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Cellular host factors for SARS-CoV-2 infection

Jim Baggen, Els Vanstreels, Sander Jansen and Dirk Daelemans ✉

The coronavirus disease 2019 (COVID-19) pandemic has claimed millions of lives and caused a global economic crisis. No effective antiviral drugs are currently available to treat infections of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The medical need imposed by the pandemic has spurred unprecedented research efforts to study coronavirus biology. Every virus depends on cellular host factors and pathways for successful replication. These proviral host factors represent attractive targets for antiviral therapy as they are genetically more stable than viral targets and may be shared among related viruses. The application of various 'omics' technologies has led to the rapid discovery of proviral host factors that are required for the completion of the SARS-CoV-2 life cycle. In this Review, we summarize insights into the proviral host factors that are required for SARS-CoV-2 infection that were mainly obtained using functional genetic and interactome screens. We discuss cellular processes that are important for the SARS-CoV-2 life cycle, as well as parallels with non-coronaviruses. Finally, we highlight host factors that could be targeted by clinically approved molecules and molecules in clinical trials as potential antiviral therapies for COVID-19.

Coronaviruses are positive-strand RNA viruses belonging to the subfamily *Orthocoronavirinae* within the family *Coronaviridae* (International Committee on Taxonomy of Viruses) and are subdivided into four genera—*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. Coronaviruses cause intestinal and respiratory infections in a variety of birds and mammals, including livestock and domestic animals.

Seven human coronaviruses (HCoVs) have been characterized, four of which cause mild respiratory infections (HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1). The emergence of two highly pathogenic betacoronaviruses in 2002 (severe acute respiratory syndrome coronavirus (SARS-CoV)) and 2012 (Middle East respiratory syndrome coronavirus (MERS-CoV)) revealed that new pathogenic coronaviruses can emerge in the human population by zoonotic transmission (reviewed in ref. [1]). In late 2019, a pathogenic coronavirus SARS-CoV-2 was first detected in Wuhan, China, causing an outbreak of severe pneumonia. This human pathogen is thought to have originated in horseshoe bats and was probably transmitted to humans through an intermediate host that remains to be identified[2]. Owing to its high contagiousness and the occurrence of asymptomatic carriers, SARS-CoV-2 rapidly spread across the globe and continues to claim human lives and obstruct social and economic activity, while vaccination programs are ongoing. The pandemic has prompted countless efforts to develop vaccines and antiviral therapies, but also many fundamental studies to better understand coronavirus biology. As cellular proviral host factors are potential antiviral drug targets, numerous studies have analysed host factor dependencies of coronaviruses, in particular SARS-CoV-2.

In this Review, we provide an overview of proviral host factors for SARS-CoV-2. After explaining the coronavirus life cycle, we discuss the cellular receptors and proteases that are required for SARS-CoV-2 entry. As only few of the identified proviral host factors have been assigned to specific post-entry stages of the life-cycle, these stages are not discussed separately. Instead, we summarize the wealth of information on proviral SARS-CoV-2 host factors that has been produced by genome-wide functional genetic screens and interactome analyses and discuss their roles in cellular processes. Finally, we highlight host factors that may serve as targets for antiviral therapies against COVID-19.

The coronavirus life cycle

The SARS-CoV-2 virion is an enveloped and pleomorphic particle with a diameter of around 60–100 nm (ref. [3]). The helical nucleocapsid contains the ~30 kb single-stranded RNA genome packaged by the nucleocapsid (N) protein[4]. It is surrounded by the viral envelope, which contains the spike (S), membrane (M) and envelope (E) proteins. Coronavirus entry into cells is mediated by S, a homotrimeric class-I fusion protein (reviewed in ref. [5]). The dual role of S is to bind to cellular receptors on target cells and to fuse viral and cellular membranes, triggered by proteolytic cleavage of S by host proteases. Coronaviruses enter cells either through endocytic pathways or by directly fusing with the plasma membrane, depending on the availability of cellular proteases (reviewed in refs. [6,7]) (Fig. 1). After fusion, the nucleocapsid is released into the cytoplasm, after which the genomic RNA uncoats by dissociation from N and is subsequently translated.

Coronaviruses have capped and polyadenylated genomes that contain multiple open reading frames (ORFs), flanked by 5′ and 3′ untranslated regions. The SARS-CoV-2 genome contains at least 11 ORFs[8], encoding 16 non-structural proteins (nsp1–16), 4 structural proteins (S, E, M and N) and a set of putative accessory proteins (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8 and ORF9b)[9]. Although coronavirus accessory proteins are non-essential for replication in vitro, these proteins are believed to be required for virulence in vivo (reviewed in ref. [10]). Viral gene expression starts with the translation of *orf1a* and *orf1b* directly from the positive-strand RNA genome pp1a is encoded by ORF1a, whereas pp1ab is formed by continuous translation of ORF1a and ORF1b through a programmed −1 ribosomal frameshift (regulated by host proteins such as SHFL), which determines the stoichiometry between pp1a and pp1ab. These polyproteins are processed by viral proteases that are located within nsp3 (PL^{pro}) and nsp5 (M^{pro} or $3CL^{pro}$), yielding the 16 replication proteins nsp1–16 (ref. [11]). nsp1 shuts down host translation and promotes host mRNA degradation, whereas nsp2–16 establish the viral replication–transcription complex (RTC). nsp2–11

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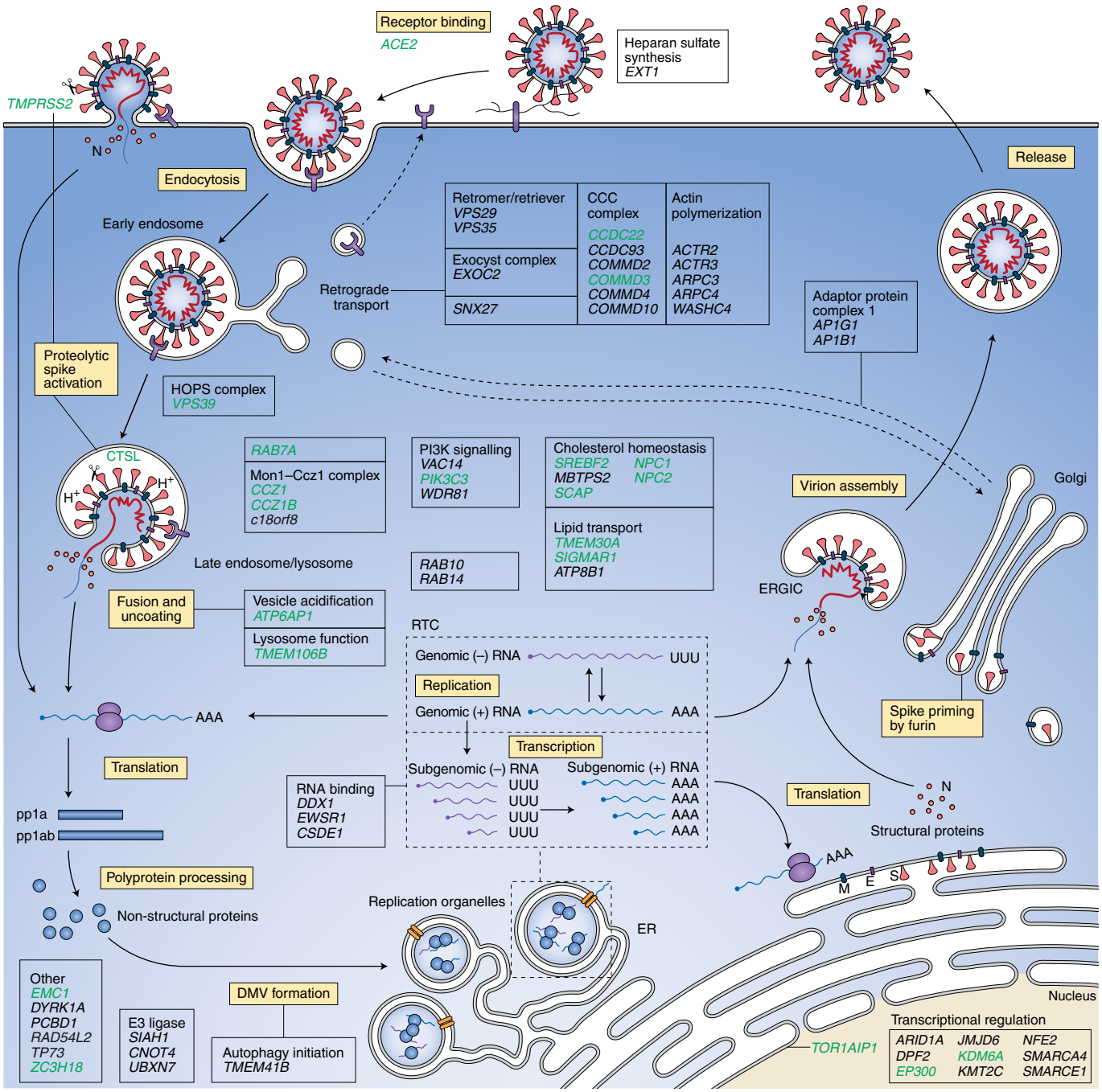


Fig. 1 | Key proviral host factors in the SARS-CoV-2 replication cycle. After SARS-CoV-2 particles attach to the target cell by interacting with receptor(s), cleavage of the S protein by cell-surface proteases (such as TMPRSS2) is thought to trigger fusion with the plasma membrane. Alternatively, SARS-CoV-2 can enter cells through endocytosis, after which fusion is induced by low pH and S cleavage by endosomal/lysosomal proteases (cathepsins). The N protein dissociates from the viral positive-strand (+) RNA genome, which is directly translated to form polyproteins pp1a and pp1ab. These polyproteins are autocatalytically processed into the non-structural proteins nsp1–16, which establish RTCs and remodel cellular membranes to form replication organelles. These organelles are continuous with the ER and provide an optimal environment for viral RNA replication, which mainly occurs inside DMVs. Genome replication starts with the synthesis of a negative-strand (–) copy that functions as a template for the synthesis of new positive-strand RNA genomes, which may enter more rounds of translation or are incorporated into new virions. Discontinuous transcription of the positive-strand genomic RNA yields subgenomic negative-strand RNAs, which function as templates for the synthesis of subgenomic positive-strand RNAs that encode structural and accessory proteins. Nascent viral RNAs exit DMVs through a transmembrane pore to reach sites of translation or virion assembly. Genomic positive-strand RNA, encapsidated by N, as well as the structural proteins S, M and E, assemble at the ERGIC, at which new virions form by budding into the lumen. Finally, progeny virions are released from the host cells. The yellow boxes indicate the different steps of the viral life cycle. The genes that were both identified as proviral SARS-CoV-2 host factors in at least two independent studies (either through pooled functional genetic screening or through individual genetic validation) and individually validated in at least one study (Supplementary Table 1) are shown. Genes that were individually validated in multiple studies and in different cell lines are highlighted in green.

FOL 26-3132 - Document 5

modulate the intracellular environment to favour viral replication, whereas nsp12–16 contain the core enzymatic functions required for RNA synthesis, including the RNA-dependent RNA polymerase (RdRp; nsp12) (reviewed in ref. [12]).

RNA replication starts with the synthesis of full-length negative-strand copies of the viral genome. These serve as templates for the production of new positive-strand genomes, which are translated to form more non-structural proteins or are packaged into newly formed virions. Moreover, discontinuous transcription of the genome generates a set of negative-strand subgenomic RNAs, which serve as templates for the synthesis of positive-strand subgenomic RNAs (reviewed in ref. [13]). These are translated to form the structural proteins and accessory proteins. RNA replication occurs in replication organelles (formed by nsp3, nsp4 and nsp6), which provide an optimal environment for replication and are thought to shield viral RNA from detection by cytosolic innate immune sensors[14]. These organelles are endoplasmic reticulum (ER)-derived perinuclear interconnected membrane structures that contain double-membrane vesicles (DMVs), in which RNA synthesis occurs[15]. Nascent viral RNA molecules escape from these DMVs through a transmembrane pore[16] to reach sites of translation or virion assembly, which occurs at the ER–Golgi intermediate compartment (ERGIC)[17]. Here, newly synthesized viral genomes—coated with N protein—bud into the lumen of the ERGIC to form enveloped particles containing M, E and S proteins. Finally, progeny virions are trafficked to the cell surface for release. Although this process is poorly understood, a recent study showed that the *Betacoronavirus* mouse hepatitis virus (MHV) hijacks the lysosomal pathway for non-lytic egress of new infectious virions[18]. Each step of the viral life cycle depends on the interplay between viral components and cellular host factors, which is discussed below for SARS-CoV-2.

SARS-CoV-2 receptors

ACE2. The coronavirus S protein consists of two subunits, S1 and S2. S1 mediates receptor binding, usually through a receptor-binding domain (RBD) within S1, whereas S2 guides membrane fusion. As angiotensin-converting enzyme 2 (ACE2) was previously shown to be a crucial receptor for SARS-CoV infection in vivo[19], ACE2 was rapidly identified as a receptor for SARS-CoV-2. Structural studies revealed that the peptidase domain of ACE2 binds to the SARS-CoV-2 S RBD[20,21]. ACE2 was confirmed to be a functional receptor by showing that overexpression of human ACE2 enabled SARS-CoV-2 infection of poorly susceptible cell lines[22,23] and mice[24], which are naturally non-susceptible. ACE2 depletion inhibited SARS-CoV-2 infection of Vero E6 (ref. [25]), Huh7.5 (refs. [26,27]), Caco-2 (ref. [28]) and Calu-3 (refs. [29,30]) cells. By contrast, other studies reported that SARS-CoV-2 could infect ACE2-deficient cells[31], although this may be due to mutations in S[32]. ACE2 exists in a membrane-bound form and a soluble form, released after cleavage by ADAM17. A recent study suggested that soluble ACE2 can also promote infection by forming a complex with SARS-CoV-2 S and vasopressin[33].

Viral tropism is partly determined by the repertoire of receptors that a virus can engage. SARS-CoV-2 enters the body primarily through the respiratory tract[34] and is most abundantly detected in the airways, but also resides in the kidneys, liver, heart, brain, blood and intestines[35,36]. *ACE2* expression in specific airway cells is increased in patients with COVID-19 (ref. [37]), most likely because *ACE2* is an interferon-stimulated gene[38]. Although SARS-CoV-2 RNA was predominantly detected in airway cell types that also express *ACE2* (refs. [37,39]), a strict correlation at the single-cell level between *ACE2* expression and SARS-CoV-2 infection has not yet been demonstrated. As SARS-CoV-2 infects many organs, it is possible that other receptors contribute to viral dissemination in vivo. Recently identified SARS-CoV-2 candidate receptors that may cooperate with or act as an alternative to ACE2 are discussed below and summarized in Table 1.

Auxiliary SARS-CoV-2 receptors. Several SARS-CoV-2 receptors most likely function as cofactors that enhance entry through other receptors. It was shown that heparan sulfate (HS) binds to SARS-CoV-2 S[40–42] and that HS depletion decreases SARS-CoV-2 infection in vitro[42,43]. Thus, analogous to other viruses, SARS-CoV-2 may engage HS to facilitate initial cell attachment, increasing the likelihood of subsequent interactions with entry receptors[44]. As many viruses bind to HS as a consequence of adaptation to in vitro cultured cells, future studies should establish whether HS binding is a natural ability of SARS-CoV-2. Scavenger receptor class B member 1 (SRB1) is a cell-surface receptor that is involved in the uptake of lipids, including high-density lipoprotein (HDL). SARS-CoV-2 S1 binds to HDL, and the addition of HDL to cells enhances infection[45]. In the presence of HDL, overexpression of *SCARB1* (which encodes SRB1) stimulated infection, whereas knockdown of *SCARB1* reduced SARS-CoV-2 infection, suggesting that SRB1 mediates the cellular uptake of HDL-bound virus. A unique feature of SARS-CoV-2 that is absent in SARS-CoV is a polybasic motif (RRAR) at the S1/S2 boundary, which can be cleaved by furin[46], resulting in a C-terminally exposed RRAR peptide. Two independent studies showed that this peptide directly binds to neuropilin-1 (NRP1) and that NRP1 promotes SARS-CoV-2 infection[47,48] (Table 1). Although SRB1 and NRP1 may be able to independently support infection, the fact that these receptors enhance SARS-CoV-2 entry in the presence of *ACE2* overexpression[45,47,48] suggests that they serve as cofactors that potentiate infection through ACE2 or other receptors.

Alternatives to ACE2. Several candidate receptors were shown to enable SARS-CoV-2 infection in the absence of ACE2. The cell-surface proteins tyrosine-protein kinase receptor UFO (Axl)[31], low-density lipoprotein receptor class A domain–containing protein 3 (LDLRAD3) and C-type lectin domain family 4 member G (CLEC4G)[49] were all shown to bind to the N-terminal domain (NTD) of SARS-CoV-2 S. Moreover, their depletion in cell lines reduced SARS-CoV-2 infection and their overexpression in *ACE2*-knockout cells promoted infection, showing that these proteins are probably alternative receptors to ACE2. Basigin (also known as CD147/EMMPRIN), a broadly expressed protein that binds to various ligands, was shown to bind to the S RBD[50], although another study did not confirm this finding[51]. Notably, expression of human basigin enabled SARS-CoV-2 infection in mice. As SARS-CoV-2 cannot use mouse ACE2 (ref. [23]), these findings implicate basigin as an ACE2-independent host factor.

Other receptors. Several other candidate receptors were shown to bind to SARS-CoV-2 S and to facilitate SARS-CoV-2 infection when overexpressed, including asialoglycoprotein receptor 1 (ASGPR1), Kremen protein 1 (ref. [52]) and transferrin receptor[53]. The innate immune receptors CD209 (also known as DC-SIGN) and C-type lectin domain family 4 member M (CLEC4M; also known as L-SIGN or CD209L) bind to numerous viruses, including SARS-CoV and SARS-CoV-2 (refs. [54–57]). These receptors have been suggested to enable virus transport between target cells (*trans*-infection)[58]. Whether depletion of the above receptors in cells affects SARS-CoV-2 infection remains to be shown. Finally, several other SARS-CoV-2 receptor candidates were suggested on the basis of their direct interaction with S (Table 1).

In conclusion, several SARS-CoV-2 candidate receptors have been identified in addition to ACE2, although it remains to be established whether these receptor interactions are biologically relevant or have resulted from cell-culture-induced virus mutations. Future studies will need to evaluate whether their expression levels

FDA 26-3132 - Document 5

Table 1 | Overview of SARS-CoV-2 candidate receptors

Name	Physiological role	Evidence for its role as a SARS-CoV-2 receptor	Refs.	
			Preprint	Peer-reviewed
ACE2	Blood pressure regulation	Cryo-EM/crystal structures of ACE2 bound to the S RBD		20,21
		Enhanced infection after *ACE2* overexpression in cells		22,23
		Enhanced infection after human *ACE2* expression in mice		24,136
		Partial inhibition of infection after *ACE2* KO in cells		26
HS	Signalling, cell adhesion	Direct interaction between HS and S (EM, SPR, pull-down, glycan array, column chromatography)		40–42
		Partial inhibition of infection after enzymatic HS removal or KO of HS synthesis enzymes in cells		42,43
NRP1	Cardiovascular and nervous system development	Direct binding of NRP1 to the RRAR peptide in S (co-IP)		47
		Enhanced infection after *NRP1* overexpression in cells		48
		Partial inhibition of infection after *NRP1* KO/KD or treatment with NRP1-specific antibody in cells		47,48
SRB1	Lipid uptake receptor	Enhanced infection of cells after *SCARB1* overexpression and reduced infection after *SCARB1* KD in the presence of HDL.		45
Axl	Growth factor signalling	Direct interaction of Axl with the S NTD (pull-down, BLI) Enhanced infection after *AXL* overexpression in *ACE2*-KO cells Partial inhibition of infection after *AXL* KO in cells		31
Basigin	Spermatogenesis, vision, immune responses	Direct binding of basigin to S RBD (SPR, ELISA, co-IP, EM) Enhanced infection after human *BSG* expression in cells and mice Partial inhibition after human *BSG* KD in cells		50
LDLRAD3, TMEM30A and CLEC4G	Unknown, flippase, glycan binding	Direct interaction with the S NTD (Co-IP) Enhanced infection after overexpression in cells Partial inhibition of infection after KD in cells	49	
CD209 and CLEC4M	Cell adhesion and pathogen recognition	Direct interaction with S (pull-down, dot blot, SPR, EM)	54,55	56,57
		Enhanced infection after overexpression in cells	54	
ASGPR1 and Kremen protein 1	Glycoprotein homeostasis/apoptosis	Direct interaction with S (co-IP) Enhanced infection after overexpression in cells	52	
Transferrin receptor (TfR)	Iron transport	Direct interaction of TfR with S and ACE2 (ELISA, SPR) Enhanced infection after human *TF* expression in mice	53	
HAVcr-1 (also known as KIM1 and TIM1)	Immune system regulation	Direct interaction between HAVcr-1 and S RBD (co-IP, FRET, MST)	137	138
		Inhibition of virosome uptake by anti-HAVcr-1 antibodies or *HAVCR1* KO	137	
BiP (also known as GRP78)	Protein folding	Direct interaction of GRP78 with S (co-IP) Partial inhibition of infection by anti-GRP78 antibodies	139	
Sialic acid	Signalling/adhesion	Direct interaction with S protein (BLI)	140	

BiP, ER chaperone BiP; BLI, biolayer interferometry; co-IP, co-immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; FRET, Förster resonance energy transfer; HAVcr-1, hepatitis A virus cellular receptor 1; KD, knockdown; KO, knockout; SPR, surface plasmon resonance.

in SARS-CoV-2 target cells is sufficient to be relevant for infection. At present, none of the SARS-CoV-2 receptors identified, including ACE2, has been validated by genetic depletion in an animal model. Such studies will be required to establish the relevance of each receptor for SARS-CoV-2 in vivo.

Proteases required for SARS-CoV-2 entry

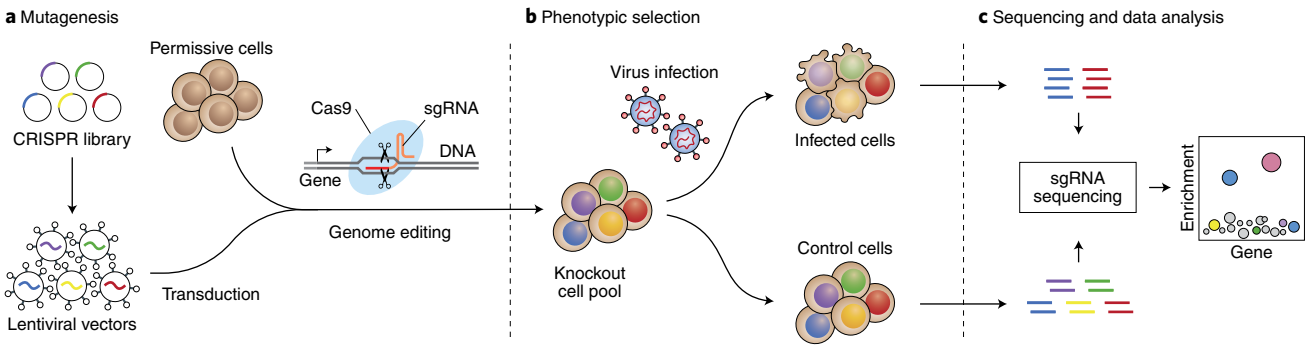
After receptor binding, the viral envelope fuses with the host cell membrane, a process guided by host protease–mediated cleavage of S. SARS-CoV-2 has remarkable flexibility in protease requirements, and the local protease availability and temperature[59] influence the viral entry route and cell tropism (reviewed in refs. [6,7]). Proteolytic activation of S occurs in two sequential steps.

The first cleavage (priming) occurs at the S1/S2 boundary for some, but not all, coronaviruses, typically during S biosynthesis at the *trans*-Golgi network of infected cells. After this cleavage, the two subunits remain bound together by non-covalent bonds and are incorporated together into assembled virions. Priming generally facilitates receptor binding and can expose hidden cleavage sites. The unique polybasic motif at the S1/S2 boundary in SARS-CoV-2 S forms a minimal furin-like cleavage site[60]. Indeed, it was shown that SARS-CoV-2 particles harbour cleaved S protein and that furin inhibition substantially reduces the amount of cleavage[46,60,61]. Furin is a ubiquitously expressed endoprotease that is mainly localized in the *trans*-Golgi. Thus, the polybasic furin cleavage site is believed to be a gain-of-function that enables systemic spread of SARS-CoV-2.

FOI 26-3132 - Document 5

Box 1 | CRISPR-based functional genetic screens for proviral host factors

Large-scale loss-of-function genetic screens are a powerful tool in the study of proviral host factors, enabling comprehensive and systematic assessment of virus–host interactions (reviewed in ref. [157]). Advantages of CRISPR-based screens are that these screens can be pooled and can be applied in any cell model, have high specificity and are based on a complete block of gene function, in contrast to RNA interference screens. To mutagenize cells, a genome-wide CRISPR library, packaged into lentiviral vectors, is introduced into a large cell population at a low multiplicity of infection to prevent multiple gene perturbations per cell (**a**). A single-guide RNA (sgRNA) directs the endonuclease Cas9 to a specific gene, at which error-prone repair of Cas9-mediated double-stranded breaks generates loss-of-function mutations, resulting in a pool of different knockout cells. A genetic screen for proviral host factors requires a permissive cell line that can be infected by the virus of interest. Phenotypic selection is usually based on the ability of a virus to induce cell death, enabling the rapid clearance of susceptible cells and expansion of virus-resistant cells (**b**). Alternatively, a fluorescent reporter virus or antibody staining of viral proteins is used to separate infected from uninfected cells[158]. Genomic DNA is extracted from selected cells, as well as unselected control cells, followed by PCR amplification and sequencing of sgRNAs (**c**). sgRNAs that are enriched in the virus-infected population as compared with the control population represent genes of which knock out prevents virus infection and are therefore proviral host factors. As cell survival mostly requires inactive virus replication, such screens generally detect genes that are involved in viral entry, translation or genome replication, but not the later stages of assembly and egress. CRISPR knockout screens cannot identify genes that are essential for cell proliferation, as their knockout depletes cells from the population. A similar strategy can be used to uncover antiviral host factors (restriction factors) by performing CRISPR-based gain-of-function screens and selecting for genes that prevent virus infection after overexpression[159].



In support of this, loss of the furin cleavage site attenuates replication in respiratory cells and reduces pathogenesis in animal models, but does not completely abolish infection[61–64]. MERS-CoV S also harbours a multibasic site that can be primed by furin, in contrast to S in the more closely related SARS-CoV, which is not primed[60].

The second cleavage (activation) occurs at the S2′ site immediately downstream of the fusion peptide and is crucial for infection of all coronaviruses. It induces conformational changes that liberate the fusion peptide, which then penetrates the host cell membrane, leading directly to membrane fusion. SARS-CoV-2 S activation at S2′ can be accomplished by transmembrane protease serine 2 (TMPRSS2) on the plasma membrane, but also by endosomal cathepsin proteases, of which cathepsin L is probably the most important[22,65]. S1/S2 priming is a prerequisite for subsequent TMPRSS2-mediated activation at the S2′ site, but not for S2′ activation by cathepsin L in TMPRSS2-negative cells[60,62,66]. These observations are consistent with the concept of early and late entry routes for coronaviruses. For SARS-CoV-2, priming by furin and activation by TMPRSS2 enable the more efficient early route through fusion at the plasma membrane. The gain of an S1/S2 multibasic site may explain the expanded tropism of SARS-CoV-2 to epithelial cells of the aerodigestive tract, which highly express TMPRSS2 (refs. [67,68]). In cells lacking TMPRSS2, S1/S2 priming is redundant and SARS-CoV-2 is endocytosed, with fusion occurring late in acidified endo/lysosomal compartments after activation by cathepsins[66].

Other proteases, such as PC1, trypsin-like proteases and cathepsins, can cleave peptides mimicking the multibasic S1/S2 site of SARS-CoV-2 in vitro[69]. Moreover, studies using pseudoviruses and selective protease inhibitors suggest that alternative proteases can cleave at S1/S2 in cells[66]. However, the biological relevance of alternative proteases in the pathogenesis of SARS-CoV-2 needs to be determined. Finally, plasmin, which is commonly elevated in people with hypertension or diabetes, may cleave at the multibasic site[70]. Similarly, S2′ cleavage can be accomplished by alternative transmembrane serine proteases, as was demonstrated for transmembrane protease serine 4 (TMPRSS4) in small intestinal enterocytes[67].

Functional genetic screens for SARS-CoV-2 host factors

Several independent CRISPR-based functional genetic screens have been carried out (Box 1) to uncover SARS-CoV-2 proviral host factors. Most were performed on a genome-wide scale[25,26,29,30,43,64,71,72], but some of the screens probed a subset of genes derived from interactome analyses[27,73] (Fig. 2). All of the screens were based on cell survival, which has the limitation that genes that are essential for cell proliferation are unlikely to be detected. Moreover, such functional screens are biased towards genes that are required for early stages of the viral life cycle and fail to detect genes that are involved in virion assembly or release. Of the many experimental parameters that differed between screens (Supplementary Table 1), the choice of cell line seems to be crucial because gene overlap was mainly observed between screens carried out in the same cell type (Fig. 2). Although lung tissue is the primary site of SARS-CoV-2 infection, multiple cell lines from different origins have been used to define host factor dependencies, mainly due to lack of a virus-induced cytopathic effect in lung-derived cell lines. Vero E6 cells are non-human African green monkey kidney-derived cells

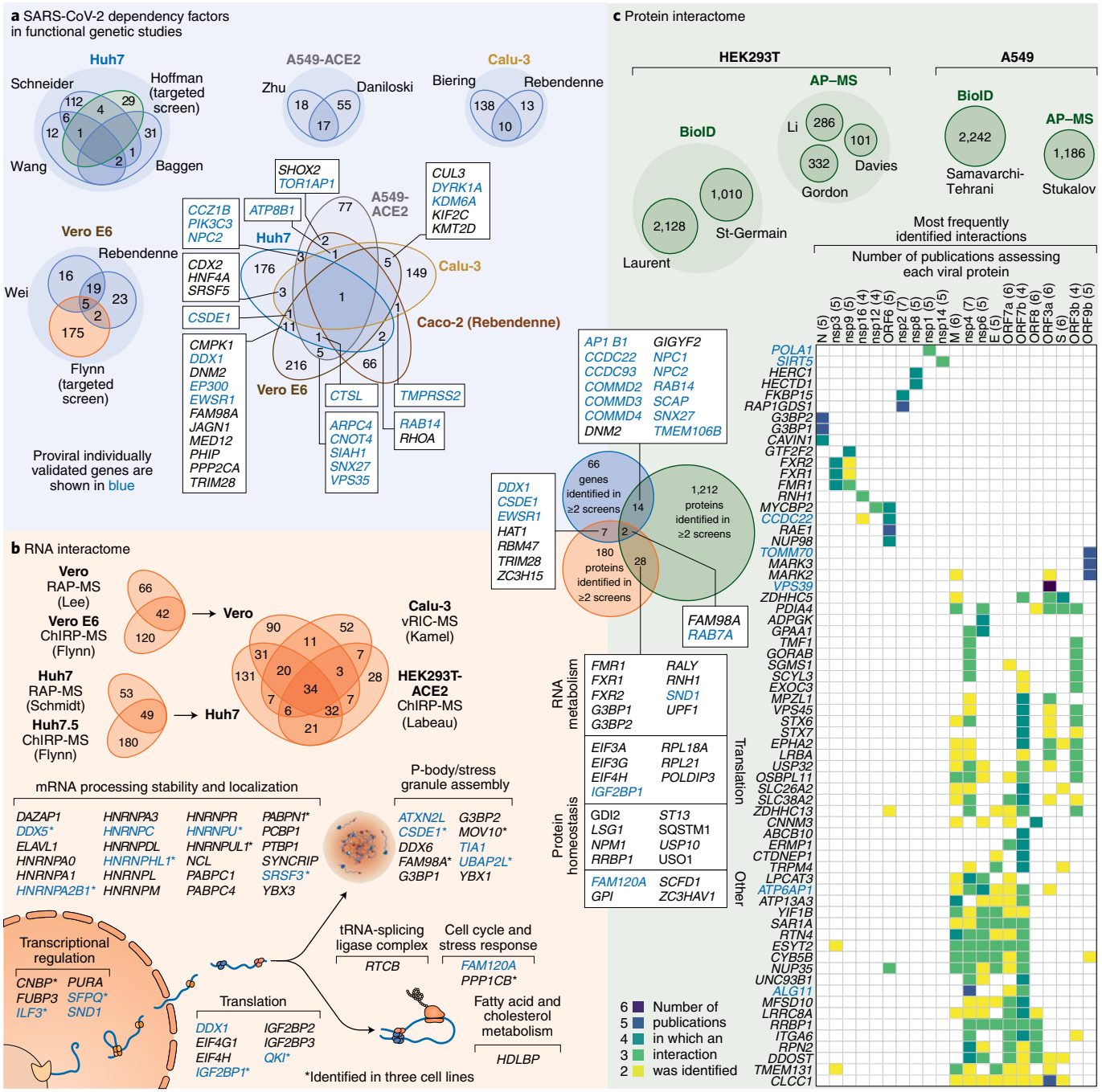


Fig. 2 | Overlapping host factors identified in functional genetic and interactome screens for SARS-CoV-2. a–**c**, Overview of human genes identified as SARS-CoV-2 proviral host factors in functional genetic screens (**a**) and genes encoding proteins identified in interactome screens as binding partners for viral RNA (**b**) or viral proteins (**c**). **a**, The number of top-ranked hits or validated proviral genes found in CRISPR-based loss-of-function studies (Zhu et al.[64], Daniloski et al.[71], Biering et al.[29], Rebendenne et al.[30], Hoffmann et al.[27], Schneider et al.[26], Baggen et al.[43], Wang et al.[72] and Wei et al.[25]). **b**, The number of proteins identified in viral RNA interactomes (Lee et al.[99], Flynn et al.[73], Schmidt et al.[100], Kamel et al.[101] and Labeau et al.[102]). Gene names are listed for proteins that meet one of the following three criteria: identified in five out of the six screens; identified in all four cell types; or identified in three cell types and functionally validated. Genes are clustered according to molecular function. **c**, The total number of cellular proteins identified in viral protein interactomes for each publication (Laurent et al.[108], St-Germain et al.[107], Li et al.[104], Gordon et al.[78], Davies et al.[103], Stukalov et al.[105] and Samavarchi-Tehrani et al.[106]) (top). Bottom, the number of publications in which a specific virus–host protein–protein interaction was detected. Genes encoding human proteins identified either in four or more publications or identified with the highest frequency for a specific SARS-CoV-2 protein (and in more than 50% of conducted screens) are shown. For viral proteins with >20 cellular interaction partners identified as such, only partners with a specificity index >2 are included (that is, the total detection number of the cellular protein divided by the number of unique viral proteins with which it was found to interact). Different isoforms were not taken into account. Genes and proteins detected in Vero cells were converted to their human orthologue. The complete gene lists are provided in Supplementary Table 2. The central Venn diagram represents the total number of genes or proteins identified in at least two screens by each approach. Genes identified by multiple orthogonal approaches are listed and clustered according to molecular function. Genes that have been individually validated as proviral factors by knockdown or knockout are shown in blue.

FOI 26-3132 - Document 5

Table 2 | Validated SARS-CoV-2 proviral host factors that serve as host factors for various viruses

Gene	Virus	Family	Function as proviral host factor	Refs.
AP1B1, AP1G1 (clathrin-associated adaptor protein complex 1)	HCV HIV	*Flaviviridae* *Retroviridae*	Virion release and cell-to-cell spread Promotes virion release through an interaction between Gag and AP-1μ	141 142
RAB7A	HIV	*Retroviridae*	Virion assembly and release	143
RAB14	HIV	*Retroviridae*	Env incorporation into assembling virions	144
VPS39 (HOPS complex)	MHV EBOV	*Coronaviridae* *Filoviridae*	Transport of virus from early endosomes to lysosomes	75 76
ATP6AP1	IAV	*Orthomyxoviridae*	Endosomal acidification is required for fusion	80
VPS29, VPS35 (retromer complex)	HCV	*Flaviviridae*	Proposed to deliver cargoes to viral replication sites through retrograde transport	87
	HPV	*Papillomaviridae*	Trafficking virus from early endosomes to the TGN	86
SNX27	HTLV	*Retroviridae*	Recycling the virus receptor GLUT1 to the plasma membrane	85
ACTR2	RSV	*Pneumoviridae*	Cell-to-cell spread through filopodia	145
SREBP pathway (*SREBF2, SCAP, MBTPS2*)	ANDV MERS-CoV	*Hantaviridae* *Coronaviridae*	Supports virus internalization into cells Suggested to have a role in DMV formation	146 147
NPC1	EBOV	*Filoviridae*	Lysosomal entry receptor	76
NPC1, NPC2	CHIKV	*Togaviridae*	Required for endocytosis or fusion	148
SIGMAR1	HCV	*Flaviviridae*	Supports early RNA replication at the ER	149
TMEM41B	ZIKV, YFV	*Flaviviridae*	Remodels host cell membranes that are required for the formation of viral replication complexes	91
PIK3C3	HBV	*Hepadnaviridae*	Supports DNA replication by inducing autophagy after HBx binding	150
CNOT4	IAV	*Orthomyxoviridae*	Required for NP ubiquitination	151
SIAH1	DENV2	*Flaviviridae*	Prevents antiviral signalling by ubiquitinating MyD88	97
JMJD6	VSIV	*Rhabdoviridae*	Inhibits the antiviral type-I interferon response	98
EWSR1	HCV	*Flaviviridae*	Supports replication through binding to the CRE in the viral RNA	152
CSDE1	CV-B3	*Picornaviridae*	Required for IRES-mediated translation	153
EMC1	SV40	*Polyomaviridae*	Mediates ER-to-cytosol transport of virions during entry	154
TOR1AIP1	HSV-1 PrV	*Herpesviridae* *Herpesviridae*	Interacts with torsin-1a, which supports nuclear egress of HSV-1 and PRV virions	155 156

Genes with known functions for other viruses that are displayed in Fig. 1 are shown. The selection of genes was determined on the basis of a literature search and includes only genes that were demonstrated (by genetic depletion) to be required for infection of other viruses, and for which the role in infection is at least partially known. ANDV, Andes orthohantavirus; AP-1μ, adaptor protein complex 1, mu subunit; CHIKV, Chikungunya virus; CRE, *cis*-acting replication element; CV-B3, coxsackievirus B3; DENV2, dengue virus 2; EBOV, Ebola virus; HBV, hepatitis B virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSV-1; herpes simplex virus; HTLV, human T-cell leukaemia–lymphoma virus; IAV, influenza A virus; IRES, internal ribosome entry site; NP, nucleoprotein; PrV, pseudorabies virus; RSV, respiratory syncytial virus; SREBP, sterol-regulatory-element-binding protein; SV40, *Macaca mulatta* polyomavirus 1; TGN, *trans*-Golgi network; VSV; vesicular stomatitis Indiana virus; YFV, Yellow fever virus; ZIKV, Zika virus.

that are permissive to a wide range of viruses in vitro. Whereas the human hepatocellular carcinoma Huh7 or colorectal adenocarcinoma Caco-2 cells are permissive to SARS-CoV-2 infection, the lung adenocarcinoma A549 cell line requires ectopic overexpression of the ACE2 receptor to support SARS-CoV-2 replication, while infection and cytopathic effect in the lung cell line Calu-3 is low. Here, we discuss genes that were consistently identified using functional genetic screens and were individually validated as proviral SARS-CoV-2 host factors (Fig. 1 and Supplementary Table 2). Although the precise role in the SARS-CoV-2 life cycle remains unknown for many of these genes, some have known functions in the life cycle of other viruses (Table 2).

The only gene identified by screens in all five cell lines tested is *ACE2* (Fig. 2). Of the other receptor candidates identified, transmembrane protein 30A (*TMEM30A*) was identified in one screen[43], whereas the HS biosynthesis gene *EXT1* was independently identified in three Huh7 screens[26,43,72]. Importantly, screens carried out in Huh7, A549 and Vero E6 cells identified *CTSL* (encoding cathepsin L), but not *TMPRSS2*, suggesting that viral entry occurred through the late endosomal route in these models. By contrast, screens in Calu-3 and Caco-2 cells[29,30] identified *TMPRSS2*, but not *CTSL*, implying that these screens probably identified factors that are involved in the early entry route.

Vesicle trafficking. Besides receptors and proteases, members of different complexes involved in vesicle biology were identified as essential for SARS-CoV-2 infection, including Rab GTPases (*RAB7A*, *RAB10* and *RAB14*). In particular, *RAB7A* was extensively validated and found in multiple screens, as were components of its dedicated Rab guanine nucleotide exchange factor, the Mon1–Ccz1 complex[74] (*CCZ1*, *CCZ1B* and *C18orf8*). *RAB7A* and *CCZ1B* were also found to support HCoV-229E and HCoV-OC43 infection[26,27,72]. Among other functions, Rab7 regulates endosomal maturation and interacts with the homotypic fusion and protein sorting (HOPS) complex, of which a subunit, the Vam6/Vps39-like protein (*VPS39*), was found to support SARS-CoV-2 infection. The HOPS complex mediates fusion of lysosomes with late endosomes or autophagosomes and, as shown for MHV[75] and Ebola virus[76] (Table 2), may cooperate with Rab7 to traffic incoming SARS-CoV-2 virions to lysosomal fusion sites. Rab7 was also found to promote cell-surface expression of ACE2 (ref. [71]). Moreover, SARS-CoV-2 ORF3A binds to the HOPS subunit Vam6/Vps39-like

protein to block autophagosome–lysosome fusion, preventing lysosomal destruction of viral components in the later stages of its life cycle[77]. Genetic screens also identified the S1 (*ATP6AP1*) and A (*ATP6V1A*) subunits of the vacuolar-ATPase proton pump[71], which were found to bind to SARS-CoV-2 nsp6 and M, respectively[78]. These factors are required for luminal acidification of vesicles and also facilitate influenza A virus entry[79,80]. *TMEM106B*, which encodes another endo-lysosomal protein, was shown in multiple studies to be required for SARS-CoV-2 but not HCoV-229E[26,43,72]. TMEM106B regulates lysosome function and interacts with the V-ATPase S1 subunit[81]. TMEM106B facilitates SARS-CoV-2 pseudovirus entry[43], further suggesting a role of the lysosomal compartment in SARS-CoV-2 entry. Furthermore, *TMEM106B* expression is elevated in SARS-CoV-2-infected airway cells from patients with COVID-19 (ref. [43]), but whether this is the cause or consequence of infection needs to be determined.

Many genes uncovered by genetic screens are involved in retrograde transport[82]. These include members of the retromer and retriever complexes (*VPS29*, *VPS35* and *VPS35L*), the retromer-associated protein sorting nexin-27 (*SNX27*) and components of the COMMD–CCDC22–CCDC93 (CCC) complex (Fig. 1). These complexes facilitate cargo enrichment and budding from the endosomal membrane to generate recycling vesicles. This budding process is driven by branched actin polymerization initiated by the Arp2/3 and WASH complexes[83], of which members were found in multiple SARS-CoV-2 screens[64,71] (*ACTR2*, *ACTR3*, *ARPC3*, *ARPC4* and *WASHC4*). Finally, multiple screens identified a member of the exocyst complex[26,72] (*EXOC2*), which interacts with the Arp2/3 complex[84] and mediates tethering of recycling and secretory vesicles to the plasma membrane. As knockout of retrograde transport genes inhibited SARS-CoV-2 pseudovirus entry and reduced cell-surface expression of ACE2 (ref. [64]), SARS-CoV-2 may require this process for receptor recycling, as shown for human T-cell leukaemia–lymphoma virus[85]. Alternatively, retrograde transport may facilitate virus trafficking to replication sites, as shown for human papillomavirus[86], or may serve to deliver cargoes to replication sites, as suggested for hepatitis C virus[87] (Table 2).

Notably, screens in Calu-3 cells did not identify any of the above-mentioned vesicle-trafficking genes, but revealed two subunits of clathrin-associated adaptor protein complex 1 (*AP1B1* and *AP1G1*)[29,30], which mediates transport between the *trans*-Golgi network and endosomes (Fig. 1). These genes were shown to support SARS-CoV-2 entry without affecting ACE2 expression on the cell surface[30] and may be required for the correct localization of other proviral factors that are involved in TMPRSS2-mediated entry.

PI3K signalling. Genetic screens also identified members of the phosphatidylinositol 3-kinase (PI3K) pathway (Fig. 1) as host factors for SARS-CoV-2, as well as for HCoV-229E and HCoV-OC43 (refs. [26,43,64,71,72]). These include PI3K type 3 (*PIK3C3*) and PI3K regulatory subunit 4 (*PIK3R4*), core subunits of a complex that produces phosphatidylinositol 3-phosphate (PI3P) and regulators of this complex (*WDR81* and *WDR91*)[64,71]. Screens also uncovered protein VAC14 homologue (*VAC14*)[26,72], which regulates the conversion of PI3P to phosphatidylinositol 3,5-bisphosphate. This signalling pathway is involved in a wide range of cellular functions, including endosomal maturation, retrograde transport and autophagy initiation (reviewed in ref. [88]). PI3K signalling may therefore facilitate SARS-CoV-2 infection by initiating the vesicle-trafficking processes discussed above, or by activating the autophagy machinery. Indeed, multiple studies showed that SARS-CoV-2 infection depends on transmembrane protein 41B (*TMEM41B*)[26,43,89], which is involved in the early stages of autophagosome formation[90]. *TMEM41B* is also required for HCoV-229E, HCoV-OC43 and HCoV-NL63 (refs. [26,43]); SARS-CoV; and MERS-CoV[89], and was recently shown to facilitate flavivirus replication complex formation[91] (Table 2). As genes required for later stages of autophagosome formation were not identified, it is possible that SARS-CoV-2 specifically hijacks early components of the autophagy machinery, including PI3K signalling, to support its life cycle.

Lipid homeostasis. Several genes identified in genetic screens with SARS-CoV-2 control cholesterol synthesis[26,72]. Sterol-regulatory-element-binding protein 1 and 2 (*SREBF1* and *SREBF2*) are transcription factors that upregulate enzymes required for fatty acid and cholesterol synthesis. Their activity is regulated by sterol-regulatory-element-binding protein cleavage-activating protein (*SCAP*) and endopeptidase S1P and S2P (*MBPTS1* and *MBPTS2*)[92], which were all identified in genetic screens. *SREBF2* was also found to support the replication of HCoV-229E, HCoV-OC43 and HCoV-NL63 (ref. [26]). Moreover, SARS-CoV-2 was found to require the late endosomal and lysosomal proteins Niemann–Pick C1 and C2 (*NPC1* and *NPC2*), which transport cholesterol from the lysosomal lumen into the lysosomal membrane[93]. Although these genes probably have indirect roles in infection, NPC1 can also directly function as a viral receptor, as shown for Ebola virus[76] (Table 2). Recently, 25-hydrocholesterol—which broadly inhibits the fusion of enveloped viruses by depleting cholesterol from the plasma membrane[94]—was shown to inhibit SARS-CoV-2 S-mediated fusion, suggesting a role of cholesterol in viral entry. Genetic screens also identified two components (*TMEM30A* and *ATP8B1*) of a P4-ATPase flippase complex that transports aminophospholipids from the outer to the inner leaflet of various membranes[29,30,43,64]. Moreover, the lipid transport protein sigma non-opioid intracellular receptor 1 (*SIGMAR1*) was found to bind to nsp6 in an interactomic screen[78]. As all steps of the viral life cycle are membrane-associated, cholesterol and other lipids could have various roles in SARS-CoV-2 infection, including innate immune system suppression, as shown for other viruses[95].

Other proviral host factors. Other identified SARS-CoV-2 host factors are involved in transcriptional regulation, including transcription factors, histone-modifying enzymes and members of the SWI/SNF complex[25,26,64,71] (Fig. 1). Moreover, screens identified several components of E3 ubiquitin ligases[25,71]. These genes may indirectly affect virus replication by regulating expression levels of other proviral or antiviral factors. For example, *EP300* and *HMGB1* enhance the expression of ACE2 on the cell surface[25,30,96]. E3 ubiquitin–protein ligase SIAH1 (*SIAH1*) and JmjC-domain-containing protein 6 (*JMJD6*) were previously shown to support infection of dengue virus[97] and vesicular stomatitis Indiana virus[98], respectively, by promoting the degradation of antiviral proteins (Table 2).

Together, functional genetic screens uncovered numerous proviral genes that are probably required for SARS-CoV-2 entry, translation or replication (Box 1). Notably, viruses used in two screens contained deletions near the polybasic cleavage site in S[43,64], whereas the sequences of the passaged viruses used in screens in the other studies were not specified[25,26,29,30,71,72]. Such deletions prevent TMPRSS2-mediated S activation and force virus entry through the endocytic route[64]. Thus, future studies with wild-type SARS-CoV-2 may be useful to extend the compendium of SARS-CoV-2 proviral host factors.

Viral RNA and protein interactomes

Interactome screens probe for cellular factors interacting with viral RNA and/or proteins and can therefore provide valuable insights into pathways that facilitate or inhibit the viral life cycle. These screens are complementary to functional genetic screens as they can also detect cell-essential factors. In brief, RNA interactome screens involve SARS-CoV-2 infection of cells, followed by RNA–protein cross-linking and identification of interacting proteins[73,99–102]. The SARS-CoV-2 RNA interactome was determined in multiple cell lines

FOL 26-3132 - Document 5

(Fig. 2) using different cross-linking techniques: formaldehyde and ultraviolet light. Ultraviolet light at 254 nm (RNA antisense purification coupled with mass spectrometry (RAP–MS))[99,100] is a more specific RNA–protein cross-linker than formaldehyde (comprehensive identification of RNA-binding proteins (chIRP-MS))[73,102], as it does not efficiently cross-link protein–protein interactions. An even more specific cross-linking technique (viral RNA interactome capture (vRIC)-MS)[101] applies 365 nm ultraviolet light after treatment with a transcription inhibitor and the photoactivatable nucleotide analogue 4-thiouridine to ensure exclusive cross-linking of 4-thiouridine-containing viral RNA. The SARS-CoV-2 protein interactome was mapped in HEK293T/17 and A549 cells using affinity-tag purification (affinity purification (AP)–MS)[78,103–105] or pull-down of tagged proteins using proximity biotinylation (BioID)[106–108]. In contrast to RNA interactomes, protein interactomes were determined by expressing individual viral proteins in uninfected cells. This approach requires prudence during interpretation, as well as validation, before making claims about virus–host interactions, as individually expressed viral proteins may localize differently and lack the context of true infection. Although these screens vary in the set of probed SARS-CoV-2 proteins, integrating the results of these studies generates a robust cellular interactome for 21 SARS-CoV-2 proteins (Fig. 2 and Supplementary Table 2).

Viral RNA interactome. When the viral RNA is released into the cytoplasm, it hijacks cellular RNA-binding proteins involved in all stages of the mRNA life cycle to achieve the arduous task of regulating translation and replication in the hostile environment of the host cell. A total of 95 proteins, representing a broad range of RNA-related functionalities, were found to bind to SARS-CoV-2 RNA in at least three different cell types, whereas 34 proteins were identified in all four cell types (Fig. 2). Flynn et al. reported a significant bias towards antiviral factors in their viral RNA interactome[73], which may explain the limited overlap between RNA interactomes and proviral factors identified in functional screens. Nevertheless, 17 of these 95 frequently detected interactors were functionally validated as proviral host factors for SARS-CoV-2 (Fig. 2).

A strong interplay was detected between SARS-CoV-2 RNA and ribonucleoprotein granules such as paraspeckles, P-bodies and stress granules. Similarly, protein families such as cold-shock domain proteins, eukaryotic translation initiation factors (*EIF3/4*) and heterogeneous nuclear ribonucleoproteins showed extensive viral RNA interaction. Several heterogeneous nuclear ribonucleoproteins have known functions in the life cycle of coronaviruses and other positive-strand RNA viruses, facilitating RNA synthesis and translation[109]. This overlapping SARS-CoV-2 RNA interactome further consisted of proteins that are involved in a plethora of cellular functions, such as transcriptional regulation, mRNA processing, stress response, fatty acid metabolism and the tRNA-splicing ligase complex[101] (Fig. 2).

SARS-CoV-2 relies completely on the host translation machinery to translate its capped and poly-adenylated genome. Thus, unsurprisingly, many RNA interactome studies contained factors that are involved in the recognition of the poly(A) tail (*PABPC1*, *PABPC4* and *PABPCN1*) and components of eukaryotic initiation factors (eIF) responsible for cap binding (eIF4F subunits) or subsequent connection to the ribosome (eIF3), as well as some 40S ribosome subunits. Remarkably, eIF4E was not retrieved in any screen, suggesting that SARS-CoV-2 translation is eIF4E-independent, similar to other capped positive-strand RNA viruses such as Sindbis virus[110]. In this regard, the identification of La-related protein 1 (*LARP1*) in the interactome of both SARS-CoV-2 RNA[73,99,100] and the N protein[78] is of special interest. LARP1 regulates the stability and translation of cellular mRNAs containing an oligopyrimidine (TOP) motif by binding to this motif and the adjacent cap, blocking the access of eIF4E. As LARP1 was validated as an antiviral factor binding to the 5′-leader of SARS-CoV-2, which contains a TOP-like motif, this may explain why eIF4E is absent from the viral RNA interactome.

Box 2 | Cellular antiviral factors that limit SARS-CoV-2 infection

As obligate intracellular parasites, viruses need to evade the host's antiviral responses to successfully establish infection. To trigger the innate immune response during infection, host cells rely on pattern recognition receptors (PRRs), such as Toll-like receptors and RIG-I-like receptors[160], to recognize specific pathogen-associated molecular patterns (PAMPs). For RNA viruses, the main intracellular PAMP is double-stranded RNA formed during viral replication, which can be recognized by cytosolic receptors, such as RIG-I, MDA-5 and PKR[161]. Although PKR activation mainly results in translational shut-off and stress granule formation, RIG-I and MDA-5 PAMP–PRR complexes bind to the mitochondrial antiviral-signalling protein (MAVS) to activate the transcription factors NFκB and interferon regulatory factors (IRFs), inducing the expression of type-I interferons (IFNs) as well as a range of other cytokines[162]. After binding to their cognate receptors, IFNs trigger the expression of several hundred IFN-stimulated genes performing various molecular functions in an effort to control viral dissemination. Like all viruses, SARS-CoV-2 has evolved several mechanisms to evade this antiviral signalling cascade.

The SARS-CoV-2 interactome captures this interplay between innate immune response and virus, encompassing both sides of the battle: the cellular effort to detect and eliminate the infection, as well as the molecular mechanisms evolved by the virus to escape and inhibit the immune response. As such, these interactomes exposed a strong interplay with stress granules (SGs), which have a pivotal role in establishing an antiviral state by limiting viral RNA translation and serving as an antiviral and apoptotic signalling hub. Viruses can inhibit their formation by sequestering or degrading stress granule components such as Ras GTPase-activating protein-binding protein (G3BP) 1 and 2 (ref. [163]), which were consistently detected in the SARS-CoV-2 RNA and N protein interactome, suggesting that SARS-CoV-2 also targets G3BP1 and G3BP2 to disrupt or deregulate SG formation[164]. Other SARS-CoV-2 proteins that have been shown to interfere with cellular antiviral responses are ORF9b and ORF6. ORF9b targets the mitochondrial import receptor subunit TOM70, which promotes MAVS activity by bridging MAVS to downstream proteins TBK1/IRF3 (ref. [165]). TOM70 was identified in several ORF9b interactome screens, and this interaction was shown to suppress MAVS-mediated type-I IFN signalling[166]. Finally, further downstream in the IFN signalling cascade, ORF6 was shown to bind to the nuclear pore complex component Nup98–Rae1 to disrupt the nuclear import of transcription factors STAT1 and STAT2 (ref. [167]), therefore impairing IFN-stimulated gene expression and further enabling SARS-CoV-2 infection.

Viral protein interactome. Of the cellular proteins found to interact with a specific SARS-CoV-2 protein (Fig. 2), a handful were identified in all of the screens conducted by at least five different research groups for a specific viral protein (G3BP1, G3BP2, TOMM70, RAE1, VPS39, MARK2 and MARK3). *G3BP1*, *G3BP2*, *TOMM70* and *RAE1* encode factors that are involved in host antiviral responses and are discussed in more detail in Box 2. The HOPS subunit Vam6/Vps39-like protein (VPS39) interacted consistently with ORF3a, as discussed above. ORF9b bound to

Table 3 | Druggable SARS-CoV-2 host factors

Protein	Gene	Therapeutic strategy	Clinical stage COVID-19
Angiotensin-converting enzyme 2	*ACE2*	Decoy receptor Pseudoligands; antibodies: TD-139, ensovibep Expression regulation: isotretinoin, RAAS inhibitors	I/II II/III III
Basigin	*BSG*	Antibody: meplazumab	II/III
HS		Lactoferrin	II/III
Transmembrane protease serine 2	*TMPRSS2*	Inhibitors: camostat mesylate, nafamostat, bromhexin, α1-antitrypsin, aprotinin	III
		Antiandrogens: bicalutamide, isotretinoin, enzulatamide	III
Cathepsin	*CTSL, CTSB, CTSD, CTSK*	Inhibitors: teicoplanin, MDL 28170, ONO 5334, VBY-825	Preclinical
Plasmin	*PLG*	Inhibitor: tranexamic acid	III
Furin	*FURIN*	shRNA: vigil	Preclinical
Anoctamin-6/transmembrane protein 16F (TMEM16F)	*ANO6*	Inhibitor: niclosamide	III
Elongation factor 1-alpha 1	*EEF1A1*	Inhibitor: plitidepsin	III
V-type proton ATPase catalytic subunit A	*ATP6V1A*	Inhibitor: biphosphonates (etidronic acid, alendronic acid)	Preclinical
Dual-specificity tyrosine-phosphorylation-regulated kinase 1A, phosphatidylinositol 4-kinase beta, activin receptor type-1B	*DYRK1A, PI4KB, ACRV1B*	Inhibitor: fostamatinib (not specific; anti-inflammatory)	III
Histone deacetylase 9	*HDAC9*	Inhibitors: romidepsin, belinostat, panobinostat, vorinostat	Preclinical
1-Phosphatidylinositol 3-phosphate 5-kinase	*PIKFYVE*	Inhibitor: apilimod	II
Sigma non-opioid intracellular receptor 1	*SIGMAR1*	Inhibitor: fluvoxamine, naltrexone (anti-inflammatory)	III
Calcium-transporting ATPase type 2C member 1	*ATP2C1*	Inhibitor: sevoflurane, isoflurane (anti-inflammatory)	III

Genetically validated SARS-CoV-2 host factors for which inhibitors are under clinical investigation for COVID-19 or approved/in advanced clinical stage for alternative indications and amenable for drug repurposing. For COVID-19 therapeutic strategies in which more than one agent is under investigation, the clinical stage of the most advanced agent is given. When the inhibitor is expected to merely have an anti-inflammatory effect rather than a direct antiviral effect, this is noted in the table. Inhibitors of which clinical investigations are halted were omitted. Sources: Clinicaltrials.gov (https://www.clinicaltrials.gov/ct2/home) and DGiDb (https://www.dgidb.org/). shRNA, short hairpin RNA.

microtubule affinity regulating kinase (MARK) 2 and 3, of which the latter was shown to affect the monocyte count in the blood of patients with COVID-19 (ref. [111]). Although MARK2 is upregulated in SARS-CoV- and MERS-CoV-infected cells[112], its kinase activity was found to be decreased in SARS-CoV-2-infected cells[113]. Interestingly, MARK2 is also hijacked by HIV-1 to facilitate microtubule-associated particle uncoating[114].

Several other interesting proteins identified with a high frequency are HERC1, CCDC22, ALG11 and ZDHHC5. Probable E3 ubiquitin–protein ligase HERC1 (encoded by *HERC1*) was picked up as a nsp8 binder and is a guanine nucleotide exchange factor that activates Rab proteins, several of which were functionally validated as proviral host factors. ORF6 showed robust interaction with the proviral coiled-coil-domain-containing protein 22 (*CCDC22*), a component of the CCC complex and regulator of NF-κB signalling. For glycolipid alpha-1,2-mannosyltransferase (ALG11), a direct link with coronavirus infection was lacking until now. However, the high-confidence interaction of ALG11 with nsp4 is an interesting topic for further research, as ALG11 is an ER-localized *N*-glycosylation protein, and *N*-glycosylation of MHV nsp4 has been shown to affect DMV morphology and RNA replication[115]. The most frequent interaction partner for SARS-CoV-2 S was the plasma-membrane-localized palmitoyltransferase ZDHHC5. Coronaviruses rely on post-translational palmitoylation of S for efficient virion production and S-mediated membrane fusion[116]. ZDHHC5 drives S palmitoylation and the consequent infectivity of HCoV-229E, and may be of equal importance for the palmitoylation of SARS-CoV-2 S[117].

Host factors as SARS-CoV-2 drug targets?

Agents that target proviral host factors may be attractive drug candidates because of a reduced chance of resistance development and the potential for broad action against viral variants. Several efforts to identify agents that inhibit SARS-CoV-2 by targeting a host protein have been undertaken[28,118–122]. Drug repurposing approaches that bypass costly and time-consuming drug discovery processes are of particular interest. Here, we summarize agents that are directed against rigorously validated host factors and that are currently in clinical trials for COVID-19 treatment, or that are approved drugs that are provisionally eligible for drug repurposing (Table 3).

SARS-CoV-2 receptors. Multiple strategies to hamper the interaction between SARS-CoV-2 and ACE2 are being pursued (Table 3; reviewed in ref. [123]). The use of excessive soluble ACE2 as a decoy to trap SARS-CoV-2 virus is under investigation. Other strategies aim to block the binding of the S RBD to ACE2 using pseudoligands or blocking antibodies, or to interfere with *ACE2* expression. As such, multiple clinical studies are evaluating the use of the anti-acne drug isotretinoin, which acts, at least partly, by downregulating *ACE2* (ref. [122]). Notably, ACE2 is a key enzyme of the

FOIA 20-3132 - Document 5

renin–angiotensin–aldosterone system (RAAS), which regulates blood pressure. RAAS inhibitors (angiotensin converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARBs)) are commonly prescribed hypertension drugs. Although ACEi do not directly block ACE2, studies suggested that they increase *ACE2* expression and may therefore worsen COVID-19 severity[124], raising concerns about the continuation of these drugs in patients with COVID-19 who are hypertensive. However, clinical investigations revealed no adverse effects[125,126], probably because ACEi/ARBs also fulfil anti-inflammatory and anti-oxidative functions. Thus, international recommendations are now against the discontinuation of these drugs in patients with COVID-19.

Blocking the accessibility of alternative receptors basigin (CD147) and HS is also under clinical investigation for COVID-19 treatment (Table 3).

Host proteases. TMPRSS2-mediated S activation at the plasma membrane is an attractive therapeutic intervention point. As *TMPRSS2* is not a cell-essential gene, inhibition is expected to cause negligible side effects. The inhibitor camostat mesylate, which has been approved for non-COVID-19 indications in Japan, is being investigated in multiple ongoing clinical trials, as well as alternative approved serine protease inhibitors (namostat, aprotinin). The protein α1-antitrypsin (α1AT) is a highly abundant circulating serine protease inhibitor that is part of the human innate immune system and has been shown to inhibit TMPRSS2 and SARS-CoV-2 entry[127]. Clinical trials with α1AT purified from donor blood, available as a pharmaceutical product (prolastin), are underway. As *TMPRSS2* expression is androgen regulated[128], several studies also investigated androgen-directed therapy (bicalutamide, enzulatamide, isotretinoin). Isotretinoin reduces dihydrotestosterone levels and, as such, downregulates *TMPRSS2*. As it also downregulates *ACE2*, isotretinoin may have a multifaceted impact on SARS-CoV-2 infectivity.

Inhibition of cathepsins, the main proteases required for pH-dependent endocytic entry, has also been examined as COVID-19 therapy[129]. However, the anti-malaria drugs chloroquine and hydroxychloroquine, which inhibit endosomal acidification required for cathepsin activity, were found to be unsuccessful in the clinic, possibly due to the existence of multiple entry routes, despite having proven antiviral activity in cell culture[130]. Similarly, studies on chlorpromazine, an anti-psychotic drug that inhibits clathrin-dependent endocytosis, and tamoxifen, an anti-cancer drug that inhibits endosomal acidification, were planned but put on hold. The glycopeptide antibiotic teicoplanin, a cathepsin L inhibitor that has been shown to inhibit the entry of SARS-CoV, MERS-CoV and Ebola virus in cell culture[131], was suggested as a complementary treatment option for COVID-19 (ref. [132]) but is not currently under clinical investigation.

The inexpensive, commonly used drug tranexamic acid, which may inhibit SARS-CoV-2 S priming by suppressing plasmin activation, is the subject of a planned clinical trial. Similarly, vigil, a plasmid delivery vehicle that contains a furin-specific short hairpin RNA that is in advanced clinical testing for cancer treatment, has been proposed as a COVID-19 therapy[133].

Other host factors. Only a few approved drugs against rigorously validated SARS-CoV-2 host factors involved in post-entry steps are currently available (Table 3). Histone deacetylase inhibitors are a relatively new class of anti-cancer agents that interfere with transcriptional regulation. For example, panobinostat was shown to downregulate ACE2 in cell culture[134]. However, the use of histone deacetylase inhibitors against SARS-CoV-2 remains unproven. The phosphatidylinositol 3-phosphate 5-kinase (PIKFYVE) inhibitor apilimod was shown to reduce viral replication in primary cells and ex vivo lung cultures and is currently being trialled against COVID-19 (ref. [121]). The same applies to niclosamide, an approved antiparasitic agent that targets TMEM16 proteins and inhibits S-mediated syncytium formation and viral replication[120], and plitidepsin, an anti-cancer agent (targeting elongation factor 1-alpha 1) with limited approval that shows in vitro and in vivo antiviral activity[135].

Outlook

The urgency imposed by the COVID-19 pandemic and the availability of functional genetic and interactomic technologies have led to the discovery of SARS-CoV-2 host factors at an unprecedented rate. Taken together, these studies have identified a large cadre of candidate receptors, genetic dependencies and cellular proteins that interact with SARS-CoV-2 RNA and proteins. Although the biological roles of some factors have been extensively investigated, other proviral host factors that were identified in multiple studies have not been characterized. Future studies should further unravel the roles of these cross-validated factors in SARS-CoV-2 infection. Moreover, studies in animal models will be required to assess the relevance of identified host factors in vivo. As SARS-CoV-2 will probably not be the last zoonotic coronavirus to emerge, it will be crucial to explore which druggable proviral host factors are also required by other pathogenic coronaviruses, to develop broad-spectrum antivirals in preparation for possible future epidemics.

Received: 10 May 2021; Accepted: 3 August 2021;
Published online: 1 September 2021

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Exh 26-3132 Document 6

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Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41564-021-00958-0.

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Peer review information *Nature Microbiology* thanks Valeria Cagno, Alfredo Castello and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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FOI 26-3132 - Document 5

Life Sciences 257 (2020) 118080

Contents lists available at ScienceDirect

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Computational insights into tetracyclines as inhibitors against SARS-CoV-2 M^{pro} via combinatorial molecular simulation calculations

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ARTICLE INFO

Keywords:
COVID-19
Tetracyclines
Drug repurposing
SARS-CoV-2 M^{pro}
Molecular simulation calculations

ABSTRACT

The COVID-19 pandemic raised by SARS-CoV-2 is a public health emergency. However, lack of antiviral drugs and vaccine against human coronaviruses demands a concerted approach to challenge the SARS-CoV-2 infection. Under limited resource and urgency, combinatorial computational approaches to identify the potential inhibitor from known drugs could be applied against risen COVID-19 pandemic. Thereof, this study attempted to purpose the potent inhibitors from the approved drug pool against SARS-CoV-2 main protease (M^{pro}). To circumvent the issue of lead compound from available drugs as antivirals, antibiotics with broad spectrum of viral activity, i.e. doxycycline, tetracycline, demeclocycline, and minocycline were chosen for molecular simulation analysis against native ligand N3 inhibitor in SARS-CoV-2 M^{pro} crystal structure. Molecular docking simulation predicted the docking score > −7 kcal/mol with significant intermolecular interaction at the catalytic dyad (His41 and Cys145) and other essential substrate binding residues of SARS-CoV-2 M^{pro}. The best ligand conformations were further studied for complex stability and intermolecular interaction profiling with respect to time under 100 ns classical molecular dynamics simulation, established the significant stability and interactions of selected antibiotics by comparison to N3 inhibitor. Based on combinatorial molecular simulation analysis, doxycycline and minocycline were selected as potent inhibitor against SARS-CoV-2 M^{pro} which can used in combinational therapy against SARS-CoV-2 infection.

1. Introduction

Human coronaviruses (HCoVs), such as middle-east respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV), and 2019 novel coronavirus (2019-nCoV, also called as SARS-CoV-2) that emerged from animal reservoirs, have aggravate the pandemic with high mortality and morbidity. Coronaviruses (CoVs) are positive-sense, single-stranded RNA (ssRNA) virus which contain the largest RNA genome of 27–32 kb with six to ten open reading frames (ORFs) enclosed in an enveloped protein to form spherical particles of 100–160 nm size [1,2]. The genetic material of HCoVs is highly susceptible to frequent recombination process which has been suggested to direct the establishment of new HCoV strains with altered virulence [3]. As a result, a total of seven main HCoVs have been recorded, viz. HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, MERS-CoV, SARS-CoV, and SARS-CoV-2; these viruses are held accountable for fatal infections in the respiratory system and other system symptoms, including diarrhea and nausea [4,5].

HCoVs belong to the Coronaviridae family of the order Nidovirales and classified into four genera (α, β, γ, and δ) [6]. Recently discovered SARS-CoV-2 belongs to the β genus which shares 79.5% sequence similarity to SARS-related coronavirus and is 96% identical at the whole-genome level to a bat coronavirus [7,8]. The genome analysis of coronaviruses, including SARS-CoV-2 genome (GenBank:MN908947.3), revealed four non-structural proteins, i.e. papain-like protease (PL^{pro}), 3-chymotrypsin-like ($3CL^{pro}$), helicase, and RNA polymerase; four structural proteins, viz. nucleocapsid (N) protein, membrane (M) protein, envelope (E) protein, Spike (S) protein, and accessory proteins [1,9–11]. Both $3CL^{pro}$ and PL^{pro} are reported in transcription and replication of the virus [6]. Remarkably, *in vitro* inhibition of these proteases by the selected inhibitors has shown antiviral activity against coronaviruses (CoVs) [12,13]. These observations concluded the $3CL^{pro}$ as the main coronavirus protease (M^{pro}) [14], which is essentially required to conduct the replication cycle of the virus [6,15]. Like in other HCoVs, SARS-CoV-2 M^{pro} digests the polyprotein at conserved sites; it starts with autolytic cleavage from pp1a and pp1ab site encoded by the

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https://doi.org/10.1016/j.lfs.2020.118080
Received 8 April 2020; Received in revised form 6 July 2020; Accepted 7 July 2020
Available online 09 July 2020
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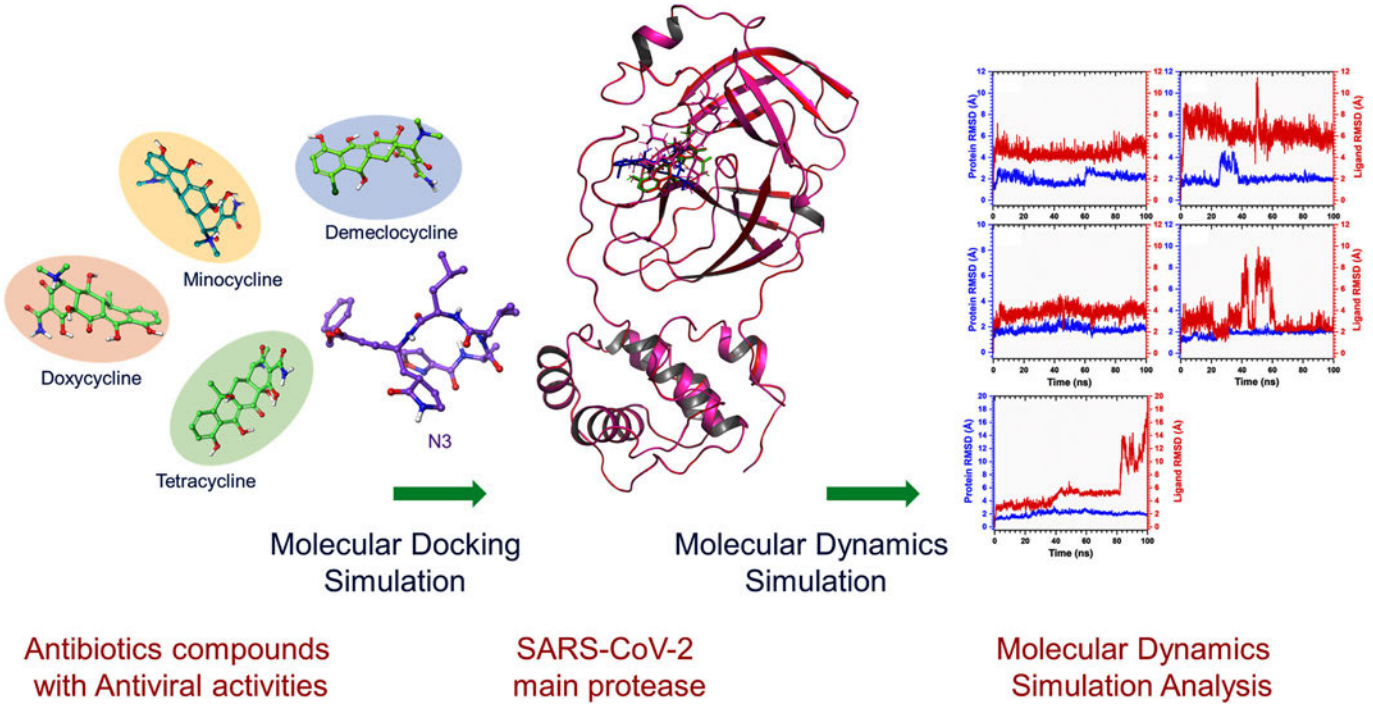


Fig. 1. Repurposing of antibiotics tetracyclines as potential inhibitors of SARS-CoV-2 main protease (M^{pro}) via combinatorial molecular simulation calculations.

virus ORF1a/b which shares 96% sequence similarity with M^{pro} of SARS-CoV strain [16,17]. To conquer coronavirus infections, the inhibition of viral M^{pro}, which is functionally important in the viral life cycle and absence of its human homologues, has been approved as conventional antiviral therapeutic strategy [12].

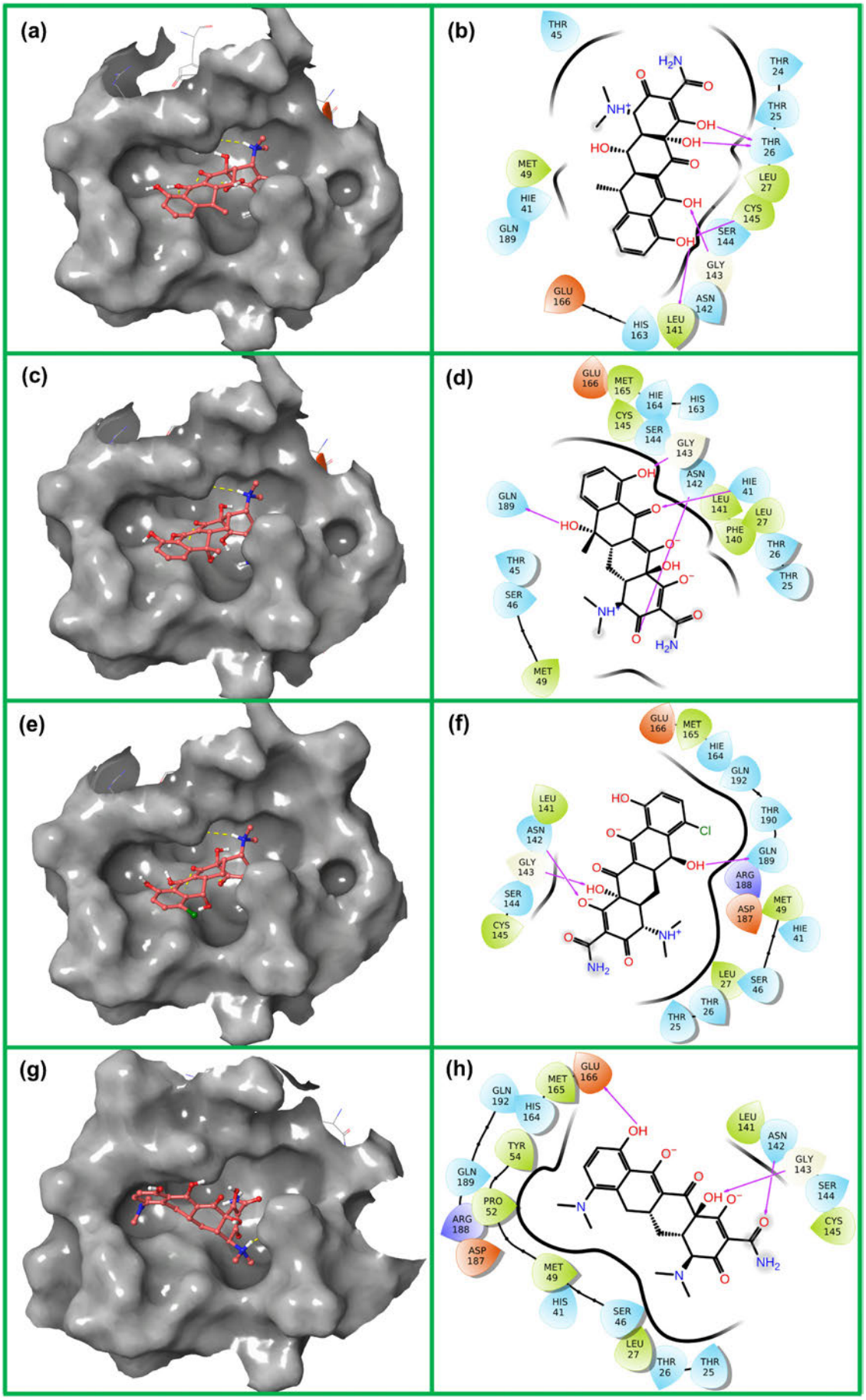
In current scenario, despite numerous efforts, no drug or vaccine is yet approved against the HCoVs and expected to take further months to years for the new interventions [11]. Recently, various M^{pro} inhibitors were demonstrated for broad-spectrum *in vitro* activities against CoVs via interacting with conserved key residues required for the substrate recognition at respective M^{pro} enzymes [18,19]. Moreover, with the urgency of SARS-CoV-2 outbreak, drug repurposing is represented as an effective drug discovery approach from existing drugs to suggestively shorten the time and condense the cost by comparison to *de novo*drug discovery strategy and randomized clinical trials [20]. To facilitate the rapid drug discovery, computational drug repurposing approaches have been established as an effective method to reveal the new indications from already known drugs with the aid of computational algorithms such as molecular docking simulation and molecular dynamics simulation to predict the drug-target interactions and binding stability, respectively [21,22]. Previously, computational drug repurposing approaches were employed to identify the potential drug candidates against viral infections, including Ebola, ZIKA, dengue, and influenza infections [23], and for HCoVs such as SARS-CoV and MERS-CoV [24–26]. Also, nonpeptidic active-site-directed inhibition of SARS-CoV M^{pro} was discovered by a combination of screening and docking methods [27]. Likewise, bifunctional aryl boronic acid compounds were discovered to inhibit the SARS-CoV M^{pro} using combination of experimental and computational methods [28]. After SARS-CoV-2 outbreak, computational drug repurposing was also applied to find the potential drugs and some of the investigations are already reported [29–31]. In area of drug repurposing, antibiotics from tetracyclines and its derivatives have been repurposed against viral infections [32,33]. These antibiotics are well designated as broad-spectrum bacteriostatic inhibitors which selectively inhibit the microbial protein synthesis only, including naturally occurring polyketide compounds (chlortetracycline, oxytetracycline, and tetracycline) and their semisynthetic derivatives (minocycline, doxycycline, and methacycline) [32]. Notably, tetracycline derivatives, i.e. doxycycline, was reported to exert antiviral effects against antimurine retrovirus [34], Chikungunya infection [33], and blockage of dengue virus replication in infected cell lines [35,36]. Besides, demeclocycline is known to exert anti-viral activity on west Nile virus (WNV) [37] and included in the list of antiviral compounds [38]. Another, broad spectrum tetracycline derivative minocycline was initially reported against human immunodeficiency virus (HIV) type 1 infection [39], and later studied for other viruses such as WNV, Japanese encephalitis virus (JEV), Simian Immunodeficiency virus (SIV), Sindbis, human T-lymphocytic virus type-1 (HTLV-1), Rabies, and Reoviruses [40]. Moreover, tetracyclines (e.g. tetracycline, doxycycline, and minocycline) which are known as zinc chelating agents were postulated to inhibit COVID-19 infection by restricting viral replication in the host [41]. Additionally, synthetic halogenated tetracycline eravacycline was demonstrated with inhibitory effect against SARS-CoV, SARS-CoV-2, MERS-CoV, and hepatitis C virus (HCV) using computational studies [42].

Hence, under the impression of tetracyclines and their derivatives inhibits viral infections, we hypothesized that antibiotics reported for antiviral activities may be helpful in the SARS-CoV-2 inhibition by blockage of its main protease, viz. M^{pro}. Thereof, we employed structure based molecular docking for selected four tetracyclines, i.e. doxycycline, tetracycline, demeclocycline, and minocycline known for antiviral activities against other viruses, with M^{pro} from SARS-CoV-2. Similar virtual screening approach was recently applied to identify the tetracycline derivatives as Flavivirus inhibitors [36]. Following, docked protein-antibiotics complexes were evaluated for the complex stability via classical molecular dynamics simulation. The binding affinity and stability of selected viral protease-antibiotics complexes were also compared to the docked complexes of protease with native ligand N3 inhibitor to generate a comparative study as mentioned in Fig. 1.

2. Methodology

2.1. Receptor and ligand collection

Crystal structure of the novel SARS-CoV-2 main protease (SARS-CoV-2 M^{pro}) was downloaded from PDB database (https://www.rcsb.



(caption on next page)

Fig. 2. 3D and 2D interaction profiles for SARS-CoV-2 M^{pro}-antibiotics; (a–b) doxycycline, (c–d), tetracycline, (e–f) demeclocycline, and (g–h) minocycline, depicting active residues around the ligand at 4 Å area in the active pocket of SARS-CoV-2 M^{pro}. In 2D interaction maps, hydrogen bond formation (pink arrows), hydrophobic (green), polar (blue), red (negative), violet (positive) and glycine (grey) interaction are also logged between the docked SARS-CoV-2 M^{pro} and selected antibiotics. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

org/) with PDB ID:6lu7 [43]. The viral M^{pro} was elucidated at 2.16 Å resolution as dimer, where each monomer contained three domains; domain I (residues 8–101), domain II (residues 102–184), and domain III (residues 201–303) connected by long interconnecting loop (residues 185–200) between the domains II and III. The catalytic dyad (His41 and Cys145) and substrate binding sites were also defined between the domains I and II in the crystal structure with N3 inhibitor [43]. Besides, 3D structures of selected antibiotics, i.e. doxycycline (CID: 54671203), tetracycline (CID: 54675776), demeclocycline (CID: 54680690), and minocycline (CID: 54675783), were also downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

2.2. Molecular docking simulation

Molecular docking simulation between the SARS-CoV-2 M^{pro} and selected antibiotics was conducted in Chimera-AutoDock Vina plugin setup to determine the interacting residues of the protein with respective ligands. Briefly, protein and ligand structures were minimized using the default parameters in structure minimization tool in USCF Chimera-1.14 [44]. Following, receptor and ligands were prepared using Dock prep tool in Chimera under default parameters, where native ligand from the crystal structure was removed, polar hydrogen atoms and charges were added. Finally, molecular docking simulations were performed using AutoDock Vina [45] plugin with default setting at the native ligand binding pocket by adopting the docking grid size of 60 × 40 × 40 Å along both three (X, Y, and Z) axes, covering all the essential residues center at −8.918, 17.918, 62.905 Å region, to provide copious space for the ligand conformations. By docking, at least 10 conformers were generated and conformer with lowest binding energy and RMSD were chosen for further analysis in free academic Maestro v12.3 (Schrödinger Release 2020-1: Maestro, Schrödinger, LLC, New York, NY, 2020). Herein, noncovalent interactions, viz. hydrogen bonding, hydrophobic, π-π interaction, π-cation interaction, positive (basic or positive amino acids), negative (acidic or negative amino acids), polar (polar amino acids), glycine (non-polar interaction), and salt bridges interactions, were calculated at cutoff radius of 4 Å under default conditions, and both 3D and 2D interaction images were generated. Similar docking methodology was also employed for the crystal structure ligand, i.e. Michael acceptor inhibitor, known as N3 inhibitor [43], to validate the docking procedure and for comparative analysis with the selected antibiotics.

2.3. Molecular dynamics simulation

The best scored poses for receptor-ligand complexes were evaluated for complex stability by 100 ns molecular dynamics (MD) simulations using free academic Desmond v5.6 module of Schrödinger-Maestro v11.8 [46] under Linux environment on HP Z2 workstation. All the MD systems for protein-ligand complexes were fabricated as an orthorhombic grid box (10 Å × 10 Å × 10 Å buffer) followed by addition of TIP4P (transferable intermolecular potential 4 point) water molecules for system minimization using System Builder tools in Desmond-Maestro interface. Later, salt and ion placement were omitted at 20 Å from the ligand and whole system was neutralized by addition of counter ions. Besides, 0.002 ps time interval was set for the anisotropic diagonal position scaling to reserve the constant pressure during MD simulation. Additionally, temperature of the system was set at 300 K coupled with 20 ps NPT reassembly at 1 atm pressure. Furthermore, system density was preserved near 1 g/cm^3 and all the calculations were conducted under default parameters. Finally, MD simulation for each complex was conducted for 100 ns interval under similar conditions. Subsequently, the simulation trajectories were analyzed by simulation interaction diagram tool in Desmond v5.6 module of Schrödinger-Maestro v11.8 [46].

3. Results and discussion

3.1. Intermolecular interaction analysis

The molecular docking algorithms have been widely used to predict the bioactive compounds or drug repurposing against various drug targetable proteins in diseases and infections [36,47]. Likewise, this study also employed the molecular docking simulation approach for the selected antibiotic tetracyclines, i.e. doxycycline, tetracycline, demeclocycline, and minocycline, to predict their instinctively potential against SARS-CoV-2 infection by its M^{pro} inhibition. All the docked drugs and N3 inhibitor showed significant docking confirmation with binding affinity energies > −7 kcal/mol at least RMSD (0 value by default in AutoDock Vina under default parameters) in the active pocket of SARS-CoV-2 M^{pro} with respect to N3 inhibitor (Figs. 2, S1). The docked SARS-CoV-2 M^{pro}-doxycycline complex was recorded with significant −7.6 kcal/mol docking score and formed five hydrogen bonds with the viral protease by active residues Thr26 (2.8 Å and 2.33 Å), Leu141 (2.33 Å), Gly143 (2.86 Å), and Cys145 (3.23 Å); additional interactions, i.e. hydrophobic (Leu27, Met49, Leu141, Cys145, Met165), polar (Thr24, Thr25, Thr26, Hie41, Thr45, Asn142, Ser144, His163, and Gln189), negative (Glu166), and glycine (Gly143), were logged in SARS-CoV-2 M^{pro}-doxycycline complex (Fig. 2a–b). Whereas, SARS-CoV-2 M^{pro} docked complex with tetracycline was logged for docking energy of −7.5 kcal/mol and formation of four hydrogen bonds with the active residues His41 (2.84 Å), Asn142 (3.33 Å), Gly143 (2.84 Å), and Gln189 (3.86 Å) was also recorded. Moreover, hydrophobic interactions (Leu27, Met49, Phe140, Leu141, Cys145, and Met165), polar interactions (Thr25, Thr26, His41, Thr45, Ser46, Asn142, Ser144, His163, His164, and Gln189), negative interactions (Glu166), and glycine interactions (Gly143) were also observed between the residues of SARS-CoV-2 M^{pro} and atoms of tetracycline (Fig. 2c–d). Similarly, SARS-CoV-2 M^{pro}-demeclocycline complex exhibited significant docking score of −7.4 kcal/mol along with formation of three hydrogen bonds at essential residues Asn142 (3.03 Å), Gly143 (2.80 Å), and Gln189 (3.74 Å) of SARS-CoV-2 M^{pro}. Additionally, the respective complex was noted for hydrophobic interactions (Leu27, Met49, Leu141, Cys145, and Met165), polar interactions (Thr25, Thr26, Hie41, Ser46, Asn142, Ser144, His164, Gln192, Thr190, and Gln189), negative interactions (Glu166 and Asp187), positive interactions (Arg188), and glycine interactions (Gly143) (Fig. 2e–f). While, SARS-CoV-2 M^{pro}-minocycline complex showed substantial binding energy of −7.1 kcal/mol followed by formation of three hydrogen bonds with the residues Asn142 (2.64 Å), Gly143 (2.54 Å), and Glu166 (2.84 Å) in the active pocket of viral protease. Other intermolecular interactions, viz. hydrophobic interactions (Leu27, Met49, Pro52, Tyr54, Leu141, Cys145, and Met165), polar interactions (Thr25, Thr26, His41, Ser46, Asn142, Ser144, His164, and Gln189), negative interactions (Glu166 and Asp187), positive interactions (Arg188), and glycine interactions (Gly143), were also observed for SARS-CoV-2 M^{pro}-minocycline docked complex (Fig. 2g–h). The substantial docking energy and formation of strong hydrogen bonds between the atoms of respective antibiotics with active residues of SARS-CoV-2 M^{pro} indicate the substantial stability of docked protease-antibiotics complexes.

Furthermore, the docked poses of the antibiotics with SARS-CoV-2

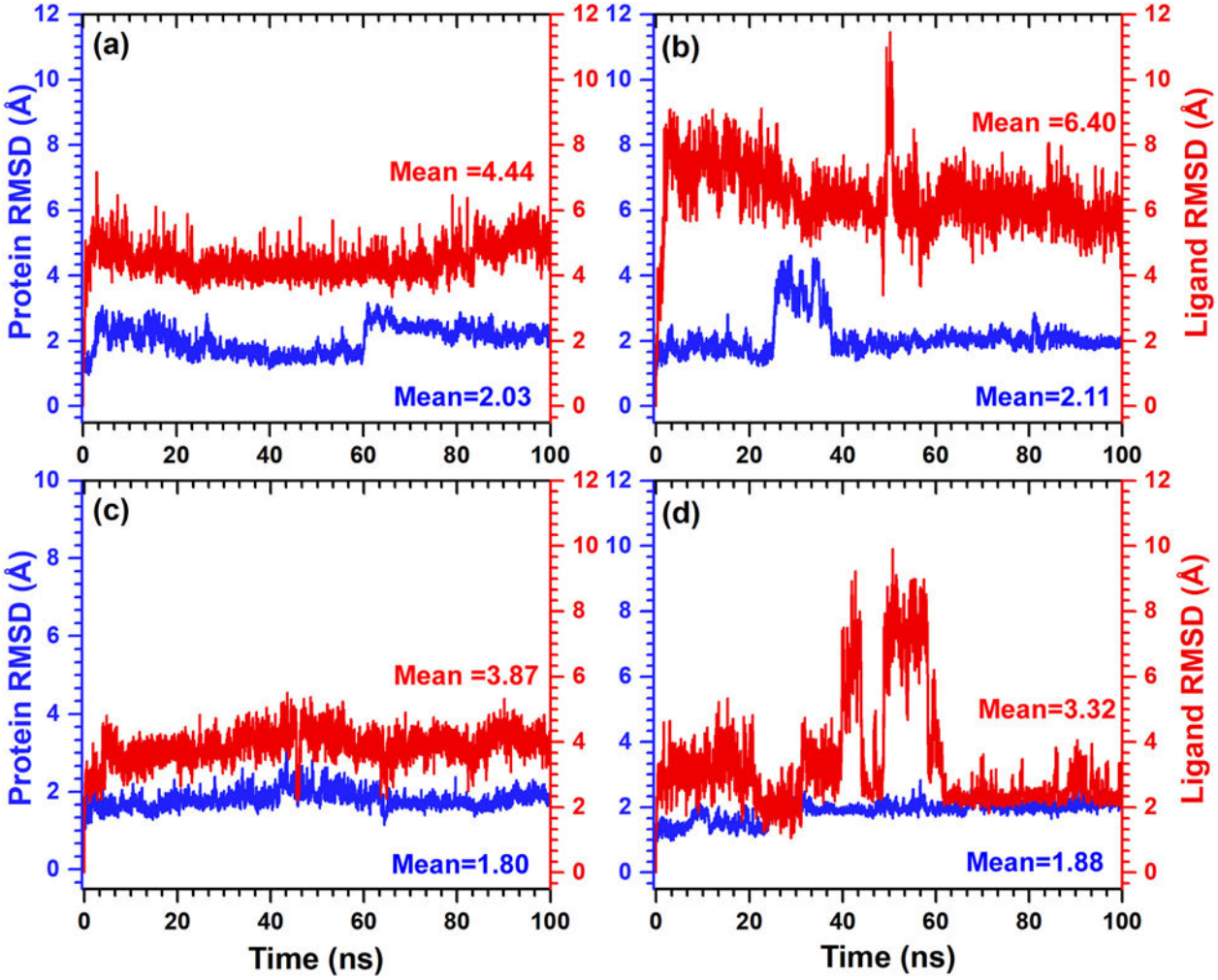


Fig. 3. Calculated RMSD values for α-carbon atoms (blue curves) of SARS-CoV-2 M^{pro} and selected ligands (red curves), viz. (a) doxycycline, (b), tetracycline, (c) demeclocycline, and (d) minocycline plotted with respect to 100 ns MD simulation time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

M^{pro} were also compared to the SARS-CoV-2 M^{pro}-N3 inhibitor docked complex (Fig. S2). The comparative docking analysis includes docking energy and intermolecular interaction profiling between the selected complexes. From molecular docking simulation analysis, N3 inhibitor (−7.6 kcal/mol) showed relatively similar docking energy by comparison to selected antibiotics (maximum docking score −7.6 kcal/mol logged for SARS-CoV-2 M^{pro}-doxycycline complex). Moreover, SARS-CoV-2 M^{pro}-N3 inhibitor complex exhibited formation of four hydrogen bonds with residues Cys145 (2.01 Å), Glu166 (2.16 Å), and Gln189 (2.30 and 3.06 Å) along with other intermolecular interactions (Fig. S2). Interestingly, in SARS-CoV-2 M^{pro}-antibiotic complexes, doxycycline also generated five hydrogen bonds and minimum of three hydrogen bonds were noted for the minocycline docked at the active pocket of viral protease. Besides, both antibiotics and N3 inhibitor exhibited significant hydrophobic, polar, negative, positive, and glycine interactions with common residues of the SARS-CoV-2 M^{pro} (Figs. 2, S2). Interestingly, selected antibiotics docked with SARS-CoV-2 M^{pro} exhibited one or more than type of interaction with the catalytic dyad (Cys145 and His41), substrate binding residues (Phe140, Leu14, Gly143, Ser144, His163, Met165, and Glu166) and other essential residues required for the substrate binding pocket (Thr24, Thr25, Met49, Phe140, Asn142, His163, Met165, Asp187, and Gln189) (Figs. 2, S2) as reported in the viral protease crystal structure with N3 inhibitor [43]. Additionally, Gln189 residue, which is absolute requirement for S1 subsite at the P1 position was logged in the interaction profile with the selected antibiotics with SARS-CoV-2 M^{pro} inhibitors [43]. As, the antibiotics were logged for intermolecular interaction with residues of active site and substrate-binding pocket located between the clefts of domain I and domain II of vial main protease, suggested to inhibit the proteolytic function of SARS-CoV-2 M^{pro}, which is required for the viral replication and pathogenesis [43]. Moreover, interacted residues of SARS-CoV-2 M^{pro} in the antibiotics docked complexes are highly conserved as substrate-recognition pocket of the CoVs, suggested the selected screened antibiotics can be employed for the development of broad spectrum of antiviral drugs against HCoVs. Hence, molecular docking simulation analysis for the screened antibiotics with respect to N3 inhibitor suggested that the selected drug molecules have the potential to inhibit SARS-CoV-2 M^{pro} by interacting with its catalytic dyad and substrate binding residues.

3.2. Molecular dynamics simulation analysis

Molecular dynamics simulation can be employed to the docked complexes to study their stability and intermolecular interaction profiling with respect to time [47,48]. In this study, docked complexes of antibiotics and N3 inhibitor with viral protease were subjected to 100 ns molecular dynamics (MD) simulation to predict the respective complex confirmation change, stability, and intermolecular interactions between the active residues of the receptor and atoms of the ligand molecules. Initially, last pose from simulation trajectory was extracted

and studied for the intermolecular interaction for the respective complexes. A maximum of three hydrogen bonds were recorded for SARS-CoV-2 M^{pro}-demeclocycline, two hydrogen bonds for SARS-CoV-2 M^{pro}-tetracycline, only one hydrogen bond in each SARS-CoV-2 M^{pro}-doxycycline and SARS-CoV-2 M^{pro}-minocycline complex against SARS-CoV-2 M^{pro}-N3 inhibitor (no hydrogen bonding) (Figs. S3, S4). These observations predicted that selected antibiotics have substantial affinity with the active pocket of SARS-CoV-2 M^{pro} by comparison to N3 inhibitor. To further verified the selected receptor-ligand complex stabilities, four properties were calculated from respective simulation trajectories, i.e. (a) root mean square deviation (RMSD), (b) root mean square fluctuation (RMSF), (c) protein secondary structure element (SSE) changes to nullify abnormal structural changes and conformational modification of docked ligands with respect to simulation time, and (d) protein-ligand contacts mapping, was performed to analyze the type of interaction between the ligand atoms and residues of the protein during MD simulation.

3.2.1. RMSD and RMSF

To quantify the degree of conformational changes in protein and ligand of the docked complexes, initially RMSD for protein (Cα, backbone, side-chain, and heavy) and fit ligand on protein were calculated from 100 ns simulation trajectories for each complex. The Cα atoms of SARS-CoV-2 M^{pro} docked with selected antibiotics and N3 inhibitor showed acceptable fluctuations (mean = 1.80 to 2.11 Å) with equilibration till end of simulation (Figs. 3, S5). Likewise, backbone, side-chain, and heavy atoms of SARS-CoV-2 M^{pro} from respective complexes were noted for stable deviations (< 3 Å), except for receptor complex with tetracycline (< 4.5 Å) showed fluctuation around 20–40 ns followed by state of equilibrium till end of 100 ns simulation (Fig. S6). Besides, RMSD of the four antibiotics fit on protein exhibited acceptable deviations (Fig. 3). Notably, tetracycline and minocycline showed maximum variation (< 10.5 Å) between 40 and 60 ns interval followed by equilibrium at 5.8 Å and 2.2 Å, respectively at the end of 100 ns simulation (Fig. 3). While RMSD for the N3 inhibitor fit on protein showed variations (~6 Å) between 40 and 60 ns and then higher deviations (~9-18 Å) after 80 ns till end of 100 ns interval (Fig. S5). These results indicate that antibiotics docked protease complexes have attained the equilibrium during 100 ns simulation while N3 inhibitor docked complex required longer simulation interval to achieve the complex stability. Thereof, based on mean of the RMSD trajectories, the selected antibiotics were concluded with significant complex stability by comparison to N3 inhibitor docked with SARS-CoV-2 M^{pro}.

Furthermore, flexibility of SARS-CoV-2 M^{pro} docked with selected compounds was analyzed to monitor the deviations in the protein residues and ligand atoms during simulation (Fig. S7). In all the complexes, acceptable RMSF values for the protein residues were recorded with respect to time where residues on the N-terminal (< 2 Å) exhibited low fluctuation compared to the C-terminal (last 10 residues with variation of > 4.5 Å). Besides, ligands were also monitored to have significant contacts with the residues in β-strands (containing active pocket of the protein), supports the integration of the docked ligands with residues on the active region of SARS-CoV-2 M^{pro} (Fig. S7). Moreover, intermolecular interactions of the ligands with secondary structure elements (α-helices and β-strands) of the protein indicate induction of slight rigidity in the protein structure. Hence, these acceptable RMSF values for protein structure and residue-ligand contact mapping suggested the structural stability of SARS-CoV-2 M^{pro} complexes with antibiotics by comparison to native ligand N3 inhibitor of crystal structure during 100 ns simulation.

Likewise, RMSF calculated for the selected antibiotics fit on SARS-CoV-2 M^{pro} with respect to simulation time suggested the acceptable deviations (< 3.5 Å) in mean structure of the ligand, expect for tetracycline (atoms at position 19 to 28) and minocycline (atoms at position 19 to 28) (Fig. S8). These variations in heteroatoms of the ligands were also concluded to contribute in relatively higher ligand fit on protein RMSD against other antibiotics during simulation. Furthermore, RMSF calculated for the N3 inhibitor fit on SARS-CoV-2 M^{pro} exhibited higher deviations (5–10 Å) (Fig. S8), which were suggested to contribute higher RMSD in the fit ligand on protein during MD simulation (Fig. S5). In conclusion, RMSD and RMSF indicate the acceptable changes in the antibiotics docked SARS-CoV-2 M^{pro} complexes by comparison to SARS-CoV-2 M^{pro}-N3 inhibitor during 100 ns MD simulations.

3.2.2. Protein secondary structure elements (SSE)

The protein SSE (α-helices and β-strands) were monitored for SSE distribution and SSE composition analysis for the respective residue index during the simulation to observed changes in the domains of SARS-CoV-2 M^{pro} docked with respective ligands (Fig. S9). The selected complexes of SARS-CoV-2 M^{pro} with antibiotics and N3 inhibitor exhibited only α-helix structures in the C-terminal region (201–303) which formed the domain III of the viral protease. While, N-terminal region showed both α-helices and β-strands (1–200) which constitute the domain I and domain III for the viral protease, defined in the crystal structure for the formation of active pocket [43]. These observations concluded the secondary structure of SARS-CoV-2 M^{pro} complexed with all the selected ligands (%total SSE = 45 ± 1.2). Interestingly, β-strand (%total 25 ± 1.1) secondary structures in the active pocket of SARS-CoV-2 M^{pro} were monitored to be significantly occupied by selected antibiotics and N3 inhibitor (Fig. S9). Furthermore, torsion angle potential plot was also studied for all the selected ligands which exhibits the relation between the torsion angle of the respective ligands to their respective potential energy [49]. The torsion angle provides the necessary information for the number of rotatable bonds in the ligand molecule (Fig. S10). Here, doxycycline, tetracycline, demeclocycline, minocycline, and N3 inhibitor were predicted with 6, 6, 7, 6, and 22 rotatable bonds in their structures. The torsion potential and histogram relationships provide a standard method to get an insight into the conformational strain introduced in the ligands during simulation, which assisted to understand the protein-bound ligand conformation. These results indicate that ligand with a smaller number of rotatable bonds showed stronger rigidity and vice-versa may contribute to generate optimum geometry for the ligand in the active pocket to attained higher stability. This can be correlated with the docking score and number for intermolecular interactions in the respective docked complexes (Figs. 2, S2). Moreover, modifications introduced in the fit ligand on protein during MD simulation were also calculated in terms of ligand RMSD, radius of gyration, intra-molecular hydrogen bonds, molecular surface area (MolSA), solvent accessible surface area (SASA), and polar surface area (PSA) (Fig. S11). These results suggested the stability of selected antibiotics at the active pocket of SARS-CoV-2 M^{pro} by comparison to N3 inhibitor.

3.2.3. Protein-ligand interaction mapping

The binding mode of ligand with the active pocket of SARS-CoV-2 M^{pro} can be further analyzed for the atomic level interactions which includes hydrogen bond formation, hydrophobic contact, ionic interaction, and salt bridge formation during MD simulation. Hence, these protein-ligand interactions were extracted from 100 ns simulation trajectories to support the respective complex stability during MD simulation. All the antibiotics by comparison to N3 inhibitor showed significant interactions with the essential catalytic residues, i.e. His41 and Cys145, in addition to other substrate binding residues in the active pocket of SARS-CoV-2 M^{pro} (Figs. 4, S12); these residues were also recorded for the intermolecular interaction with the ligands during molecular docking simulation (Figs. 2. S2). Interestingly, all the antibiotics were observed for higher number of interactions with residues in protein-ligand mapping and formation of hydrogen bond with active residues of the protein for 30% of the simulation interval with an exception in SARS-CoV-2 M^{pro}-tetracycline complex (Fig. 4). However, only Asn142 and Glu166 were predicted with substantial hydrogen boning and water bridges formation in SARS-CoV-2 M^{pro}-N3 inhibitor

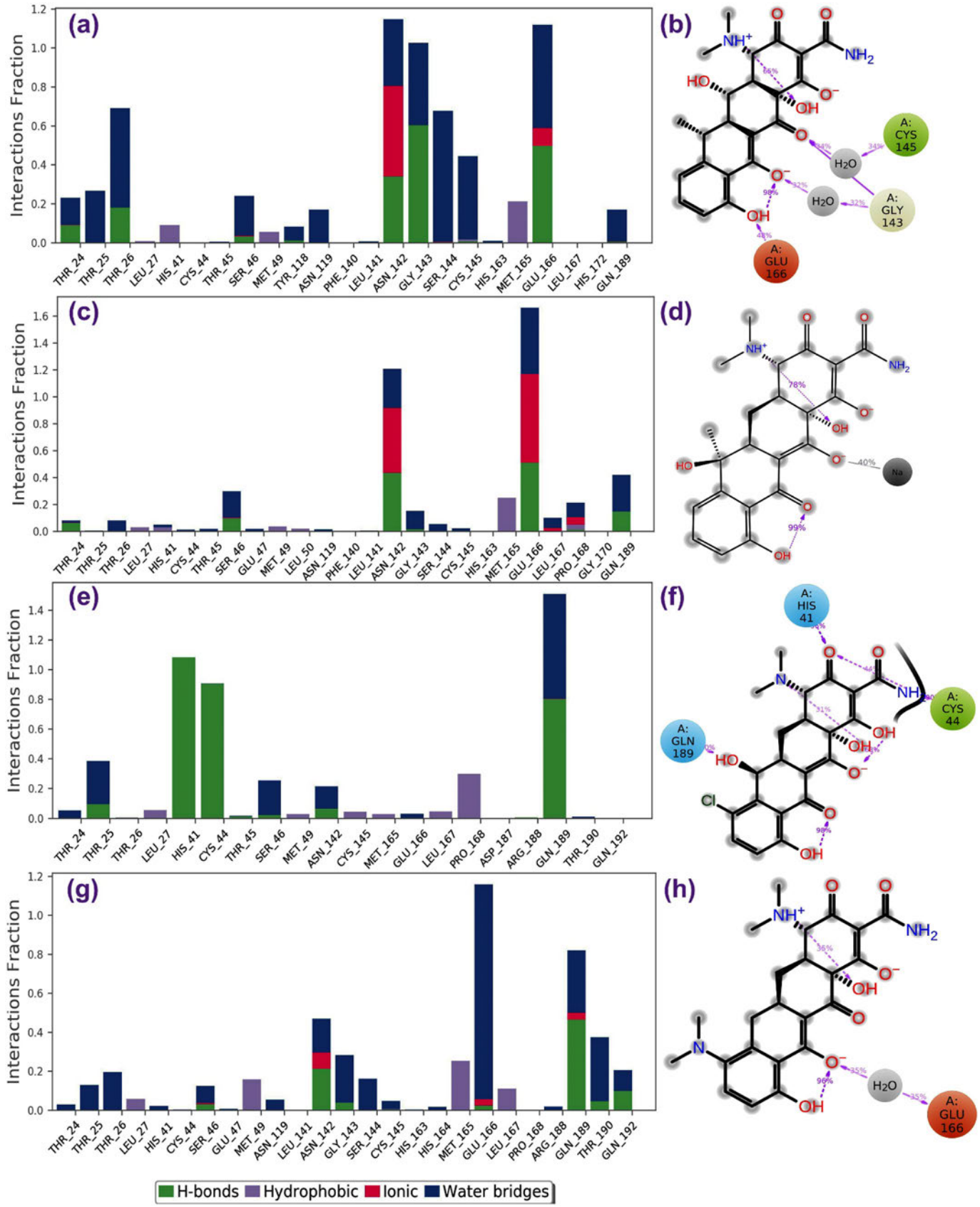


Fig. 4. Protein-ligand interactions mapping for SARS-CoV-2 M^{pro} with selected antibiotics, i.e. (a–b) doxycycline, (c–d) tetracycline, (e–f) demeclocycline, and (g–h) minocycline, extracted from 100 ns MD simulations. In 2D interaction diagram, the residues cystine (green), glutamic acid (red), histidine and glutamine (blue), and glycine (grey) exhibit the hydrophobic, negative, polar and non-polar interactions, respectively along with hydrogen bonding (pink arrow) and ionic interaction with sodium ion (black line) with the receptor are extracted at 30% of the total MD simulation interaction interval. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

complex from protein-ligand interaction mapping data analysis (Fig. S12). Furthermore, total number of contacts and their density with respect to time were also calculated (Fig. S13). Again, these plots suggested the multiple interactions of the selected antibiotics by comparison to N3 inhibitor with the essential residues (His41, Asn142, Gly143, Cys145, Glu166, and Gln189) in the active pocket of SARS-CoV-2 M^{pro}. These observations suggested the substantial protein-ligand intermolecular interactions and stability of four antibiotics by comparison to N3 inhibitor throughout the 100 ns MD simulations.

Hence, based on docking score and simulation trajectory analysis, selected four antibiotics are predicted as potential SARS-CoV-2 M^{pro} inhibitor in the order, viz. doxycycline, minocycline, demeclocycline, and tetracycline, to challenge the SARS-CoV-2 infection by comparison to N3 inhibitor. Recently combinational therapy of lopinavir with the other potent compounds against SARS-CoV-2 virus has been suggested to increase synergy and decrease the effective concentration of lopinavir [50]. For instance, triple antiviral therapy composed of interferon β-1b, lopinavir–ritonavir, and ribavirin against SARS-CoV-2 M^{pro} was reported as superior and safe against lopinavir–ritonavir alone to reduce the virus shedding, lessening symptoms, and facilitating discharge of infected patients with mild to moderate degree of SARS-CoV-2 infection [51]. In this regard, based on our computational studies, the selected antibiotics, i.e. doxycycline, minocycline, demeclocycline, and tetracycline, may be helpful as combinational therapy with lopinavir-ritonavir and ribavirin against SARS-CoV-2 infection.

4. Conclusion

This study was aimed to repurpose the antibiotics with known antiviral activity as potential inhibitor against SARS-CoV-2 M^{pro} with the aid of molecular simulation approaches. A total of four potent antibiotics, viz. doxycycline, tetracycline, demeclocycline, and minocycline were evaluated as SARS-CoV-2 M^{pro} inhibitor by comparison to native ligand of SARS-CoV-2 M^{pro} crystal structure (N3 inhibitor). Molecular docking simulation for the selected antibiotics showed docking score > -7 kcal/mol and interaction with catalytic dyad of SARS-CoV-2 M^{pro}, i.e. His41 and Cys145. These complexes were further analyzed for structural stability by 100 ns classical molecular dynamics simulation, suggested the considerable stability of SARS-CoV-2 M^{pro} docked complexes with antibiotics by comparison to N3 inhibitor of SARS-CoV-2 M^{pro} via strong multiple intermolecular interactions. From the binding mode analysis, before and after molecular dynamics simulation analysis followed by interaction profiles collected during the simulation interval supported the selected antibiotics for substantial stability on the active pocket of SARS-CoV-2 M^{pro}. In conclusion, based on docking score and molecular dynamics simulation trajectory analysis, selected four antibiotics can be used as potential SARS-CoV-2 M^{pro} inhibitor in the order, viz. doxycycline, minocycline, demeclocycline, and tetracycline to challenge the SARS-CoV-2 infection. Hence, the screened and computationally validated antibiotics can be further evaluated for *in vitro* SARS-CoV-2 M^{pro} inhibition and viral infection as single dose or in synergistic concentrations for the drug development and combinational therapy against SARS-CoV-2 infection.

Acknowledgment

This work was supported by the 2020 Yeungnam University Research Grant 220A380070.

Author contributions

Shiv Bharadwaj and Vivek Dhar Dwivedi designed the study. Shiv Bharadwaj performed all the computational experiments. Shiv Bharadwaj and Vivek Dhar Dwivedi analyzed the data. Kyung Eun Lee and Sang Gu Kang assisted in data analysis. Shiv Bharadwaj wrote the original and revised manuscript. Sang Gu Kang was responsible for the conduction and evaluation of the present work.

Data availability

The docking structures and molecular dynamics simulations trajectories are available upon request from the corresponding authors.

Declaration of competing interest

The author declares that there is no competing interest in this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.118080.

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Favorable outcome on viral load and culture viability using Ivermectin in early treatment of non-hospitalized patients with mild COVID-19 – A double-blind, randomized placebo-controlled trial.

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Abstract

Background: Ivermectin, an anti-parasitic agent, also has anti-viral properties. Our aim was to assess whether ivermectin can shorten the viral shedding in patients at an early-stage of COVID-19 infection.

Methods: The double-blinded trial compared patients receiving ivermectin 0·2 mg/kg for 3 days vs. placebo in non-hospitalized COVID-19 patients. RT-PCR from a nasopharyngeal swab was obtained at recruitment and then every two days. Primary endpoint was reduction of viral-load on the 6th day (third day after termination of treatment) as reflected by Ct level>30 (non-infectious level). The primary outcome was supported by determination of viral culture viability.

Results: Eighty-nine patients were eligible (47 in ivermectin and 42 in placebo arm). Their median age was 35 years. Females accounted for 21·6%, and 16·8% were asymptomatic at recruitment. Median time from symptom onset was 4 days. There were no statistical differences in these parameters between the two groups.

On day 6, 34 out of 47 (72%) patients in the ivermectin arm reached the endpoint, compared to 21/ 42 (50%) in the placebo arm (OR 2·62; 95% CI: 1·09-6·31). In a multivariable logistic-regression model, the odds of a negative test at day 6 was 2.62 time higher in the ivermectin group (95% CI: 1·06–6·45). Cultures at days 2 to 6 were positive in 3/23 (13·0%) of ivermectin samples vs. 14/29 (48·2%) in the placebo group (p=0·008).

Conclusions: There were significantly lower viral loads and viable cultures in the ivermectin group, which could lead to shortening isolation time in these patients.

The study is registered at ClinicalTrials.gov: NCT 044297411.

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Background

Ivermectin is an FDA-approved broad spectrum anti-parasitic agent, which was initially approved in humans in 1987 to treat onchocerciasis, awarding the discoverers the Nobel prize of Medicine in 2015. Its main activity was known for therapy against infections caused by roundworm parasites. Over the years, the spectrum was extended and included also parasitic skin infections such as scabies among others. [1]

In the last decade, several in-vitro studies have shown its anti-viral activity against a broad range of viruses, mainly RNA viruses including HIV, influenza and several flaviviruses such as Dengue virus (DENV), Zika, and West Nile Virus.[2-6] Recently ivermectin was tested in vitro against SARS-CoV-2 and showed ~5000-fold reduction (99.8%) in viral RNA after 48 hours.[7] However, it was criticized that the dosing used in the study cannot be achieved with the current approved dose.[8]

In addition, ivermectin has anti-inflammatory properties.[9] Since the excessive inflammatory response to SARS-CoV-2 is thought to be a major cause of disease severity and death in patients with COVID-19, [10] ivermectin may have further value in addition to its anti-viral properties.

With its high safety profile, ivermectin is a potential treatment against COVID-19 in its different stages. Some clinical studies have shown beneficial results regarding clinical outcomes and the length of viral shedding, however most of them are lacking a high standard of rigorous methodology.[11]

Here we conducted a double blinded randomized control trial to assess whether Ivermectin can shorten viral shedding, in non-hospitalized patients at the early stage of COVID-19 infection.

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Methods

Study design

A randomized controlled, double blinded trial to evaluate the effectiveness of ivermectin in reduction of viral shedding among mild to moderate COVID-19 patients. The study was conducted in hotels located in (Tel-Aviv, Jerusalem and Ashkelon) Israel, that have been designated as isolation facilities for mild to moderate COVID-19 patients, not requiring oxygen.

Institutional Review Board (IRB) approval was given by the Sheba Medical Center's IRB (7156/20**)**. Written informed consent was received from each participating individual before recruitment.

Study population

Patients were eligible for enrollment in the study if they were 18 years of age or older; not pregnant; with molecular confirmation of COVID-19 by RT-PCR; and with the intention to receive results within the first three days from symptoms onset. However due to the delay (three to four days) in getting results in the community, we extended the time up to seven days from symptoms onset. Since our main outcome was the change in viral shedding (as reflected by Ct value), asymptomatic cases were also included within 5 days from molecular diagnosis.

Patients were excluded if they weighed below 40kg, were with known allergy to the drugs, unable to take oral medication, participating in another RCT for treatment of COVID-19. In addition, patients who had RT-PCR results with Ct (cycle threshold) value >35 in first two consecutive were excluded. Patients with comorbidities of cardiovascular disease, diabetes, chronic respiratory disease (excluding mild intermittent asthma), hypertension, and or cancer were defined as high-risk patients.

Randomization

Randomization in a 1:1 ratio was done by computer-generated program using randomization.com (http://www.jerrydallal.com/random/randomize.htm) by Clinical Research Coordinator (CRC), blinded to the rest of

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the study team. This CRC was not requiting patients and the numbered pills bottles were available only for the physician who were requiting.

Patients assigned to the intervention arm received ivermectin in a dosage regimen according to body weight; patients weighing between 40-69 kg received four tablets (=12mg) daily and patients weighing ≥70kg received five tablets (=15mg) daily, all for three days. Patients assigned to the placebo arm received the same number and same appearance of pills per weight daily, for three days. They were guided to take the pills one hour before a meal. The investigators and patients were blinded to the assignment.

Intervention

On the day of randomization and treatment initiation, patients were tested for SARS-CoV-2 by reverse transcriptase polymerase chain reaction (RT-PCR) from nasopharyngeal (NP) swabs (day zero). Tests were then administered every two days from day six up to day 14, unless patients were discharged earlier from the isolation facilities. The protocol was amended at the beginning of September when the Ministry of Health changed the policy of isolation and allowed infected patients to leave the facility 10 days from symptom onset without further testing. At this point testing at day two and four were added to the protocol.

Sinc results of the test could have been influenced by the examiner who performed the swab and with differences between labs,[12, 13] a small number of trained practitioners were allocated to obtain the swab during the entire rial and instructed to use a uniform technique. In addition, all RT-PCR tests, including verification that patients were positive on day zero, were conducted by the same lab, at the Israel Central Virology Laboratory of the Ministry Of Health (located at Sheba Medical Center).

Patients were followed up daily by telephone until their discharge. Patients were asked whether they took the pills as guided, if they noticed any adverse effect following treatment and whether there were any follow up of symptoms.

Unexpectedly some patients who were isolated in the hotels as verified positive patients were found to be borderline or negative upon our RT-PCR test (Figure 1). Therefore,

Patients with missing data along the follow up were carried over from the last data available

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Outcomes

The primary clinical endpoint was viral clearance following a diagnostic swab taken on the sixth day (third day after termination of treatment), in the intervention group compared to placebo. Although negative PCR is defined in Israel with Ct>40, reaching this level may take a few weeks, and there is significant evidence that a non-infectious state is usually achieved at Ct level>30.[14-16] Therefore we defined a negative test at a non-infectious level as measured by RT-PCR of Ct values >30 (less than $3{\cdot}4X10^4$ viral copies)

Post-Hoc analysis: Toward the end of our study (January 2021) the central virology lab. established a Biosafety Level 3 (BSL-3) unit, allowing us the ability to culture the virus. Since positive medium of participating patients were kept in -80c, we were enabled to culture them. Thus, an end point of culture viability at days 2-6 post-intervention was added.

PCR testing

The presence of the SARS-COV-2 RNA was detected using the Seegene Allplex CoV19 detection kit, according to the manufacturer's instructions (See supplement). The test detects three viral genes: envelop (E), nucleocapsid (N) and RNA-dependent RNA polymerase (RdRp). For each sample, Ct level was defined as the Ct level of the highest viral load (low Ct).

Viral copies Determination

Determination of the copies number in the examined samples was performed by generating standard curves for each reaction, thereby enabling the conversion of the Cq values to viral genome copies, as detailed in the Supplementary methods.

In-Vitro cultures

Positive samples were stored at -80°C and were thawed for culturing on Vero E6 cells at 37°C for seven days, as detailed in the Supplementary methods.

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Statistical methods

Sample size: Based on published data from the Ministry of Health at the time of study initiation, we expected less than 10% of patients at day six show a negative RT-PCR test. With the interventional drug we expected a reduction of at least 25% in the proportion of positive cases. Hence, considering a potential decrease from 90% to 67·5% (25% decrease), with a power (1-β) of 80% at a significance level of 5% ($\alpha = 0{\cdot}05$), a minimal sample size of 96 participants in total, was required to detect a statistically significant difference. Therefore, 48 patients were needed in each study arm. To account for a loss to follow-up of 10% after 14 day, we aimed to recruit a total of 105 participants.

Statistical analysis: Statistical analysis was done by the Biostatistics and Biomathematics Unit, Gertner Institute, Sheba Medical Center, Tel-Hashomer, Israel.

Continuous variables are presented as mean ± standard deviation or as median and interquartile range. Categorical variables are presented as N (%). Differences between ivermectin and placebo groups were assessed using a Chi-square test and t-test, for categorical and continuous data respectively. Where cross tabulation frequencies were less than 5, the Fisher exact test was used. A multivariate logistic regression model was used to determine the impact of ivermectin while controlling for age, sex, weight, and being symptomatic or not on reduction of viral load on day 6^{th} as reflected by Ct level>30. Results include adjusted odds ratios (OR), and 95% confidence intervals (CI). Kaplan-Meier curves were drawn, and survival analysis conducted with log-rank test using for time to negative RT-PCR (Ct level>30) result.

Boxplots were produced in R version 4·0·2. For figure readability, viral load values were log-transformed.

For all analyses, significance was set at $p < 0{\cdot}05$. All data analyses were performed with the SAS 9·4 software (Cary, NC, USA).

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Results

From May 15th, 2020 through end of January 2021, a total of 116 patients underwent randomization, and 89 were eligible for analysis (Figure 1).

The baseline study population characteristics are detailed in Table 1. The median age of the patients was 35 years (range, 20 to 71), 22·4% equal or older than 50 years and 7·8% equal or older than 60 years. A majority of the patients were males (78·4%). Twelve (13·5%) patients had comorbidities associated with risk for severe disease [17]; 17% and 9·5% among the ivermectin and placebo groups respectively, p=0·53.

Clinical Presentation

Among the study population 83% were symptomatic. The most common symptoms, fatigue, fever, cough, headache, and myalgia were prevalent in approximately half of the study population. (symptoms detailed in Table S1-supplement). None of these variables were statistically different between the two study arms.

Study Outcome

Viral load during the study period is depicted in Figure 2. Viral load of the ivermectin group decreased faster in comparison to the placebo group at the early stage of the intervention, during days two to six. As spontaneous recovery takes place also in the placebo group the viral load is decreasing, having similar viral load since day eight.

As mentioned above, our calculations were based on negative results reflected in Ct >30. The rate of negative RT-PCR for SARS-CoV-2 at day four (one day after termination of treatment) through day ten was higher in patients receiving ivermectin but was statistically significant on days six to eight (Table 2).

In the multivariable logistic regression model, the adjusted odds ratio of negative SARS-CoV-2 RT-PCR negative test (Ct>30) at day six and eight for the ivermectin group were 2·62 (95% CI: 1·06–6·45, P=0·04) and 3·87 group (95% CI: 1·36–11·04, P=0·01) fold higher than for the placebo group, respectively. (Table 3)

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Kaplan-Meier analysis (Figure 3) adjusted to symptom onset showed the significant difference between the ivermectin and placebo arms during the course of treatment.

Clinical outcome

During the study period four patients were referred to hospitals, with three of them being in the placebo arm. The first placebo-treated patient was hospitalized for 11 days with prolonged respiratory symptoms and needed oxygen even after his discharge from hospital. The second was hospitalized for one day due to respiratory complaints. The third one was referred to hospital due to headache and dizziness and was diagnosed with sinusitis after evaluation (brain CT and MRI). In addition, one asymptomatic patient became symptomatic, which occurred in the placebo group. In the ivermectin arm, one patient was referred to hospital due to shortness of breath at the day of recruitment. He continued the ivermectin and a day later was sent back to the hotel in good condition.

Culture Positivity rate

A convenient sample of 16 samples on day of recruitment (day zero) was cultured. Ct levels ranged from 14-28 (mean 21·5±4·1), and among them 13/16 (81·2%) turned to be positive. Culture viability was tested further by available samples on days two, four and six after intervention (see details Table S2–supplement). Altogether 52 samples were cultured; viable culture in the placebo group were positive in 14 out of 29 cultures (48·2%) while among the ivermectin group, only 3/23 (13·0%) were found positive (P=0·008).

In a composite calculation, taking into account Ct values >30 together with non-viable culture, the negative results of the ivermectin group reached significance even at day four (one day after ending the treatment) with 86% negative patients compared to 59% in the placebo group (P=0·04) (see Table 2b).

Adverse events

Among the 116 intention to treat participants, 3 patients reported having diarrhea following the treatment, two (3·5%) in the ivermectin group and one (1·7%) in the placebo group. In all cases the diarrhea resolved in two days. Two patients in the placebo arm reported rash during the treatment course which subsided within one to two days.

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No other adverse effects were reported. All of the eligible 89 patients for analysis reported to be adherent to the treatment as guided.

Discussion

In this double-blind, randomized trial with mild COVID-19 patients, ivermectin significantly reduced time of viral shedding and affected viral viability when initiated at the first week after evidence of infection. Our primary endpoint was to show the benefit of ivermectin on day six (three days after ending treatment) which was achieved with 72% of samples being non-infectious (Ct>30) in comparison to 50% among the placebo group (OR 2·6). Even at day 4 (1 day after treatment end) the ivermectin group showed an OR of 2·4, although this did not reach significance, possibly due to a small sample size on that day.

The anti-viral activity was also reflected in the Kaplan-Meier curve where the effect of the drug was seen after the second day of treatment (Figure 3).

To further explore the anti-viral activity, we observed the culture viability in both placebo and ivermectin groups. This post-hoc analysis became available in our institution at the end of the study only, when the BSL-3 lab was established (January 2021). The results show the advantage of ivermectin where only 13% of samples stayed positive on days 2two to six, while 48% stayed positive in the placebo group (P=0·008).

The broad-spectrum antiviral activity of ivermectin is related to its ability to target the host importin (IMP) α/β1 nuclear transport proteins responsible for nuclear entry of cargoes of viral proteins, which in turns block the host anti-viral activity. In some viruses the viral protein (such as integrase and NS5) has been identified while in SARS-CoV-2 the protein was not identified.[18, 19] The anti-viral properties of ivermectin against SARS-CoV-2 was shown in an in-vitro model.[7] A major criticism regarding this in-vitro model was that the ivermectin concentration used was more than 35 times higher than the maximum plasma concentration after oral administration of the approved dose.[8] But higher doses may not be necessary as some models predict that the lungs achieve higher

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concentrations, up to 10-fold higher than in the serum.[20] In addition, ivermectin concentrations in blood may not reflect the activity of its other metabolites which might be the active agents.[21, 22]

In humans, several randomized control trials have been recently published. A double-blind randomized control trial conducted in Colombia by López-Medina et al., included 476 mild patients randomly assigned to receive either oral ivermectin 300 mcg/kg for five days vs. placebo.[23] The study was initially aimed to examine ivermectin as an agent which could prevent clinical deterioration when given during the early stage of COVID-19. However, the endpoint of this study was altered to be time to resolution of symptoms within 3 weeks, as the original endpoint of clinical deterioration could not be achieved due to the low number of hospitalizations in their cohort. As the authors mentioned in their limitations, reduction in the viral load or viral shedding better reflects the anti-viral activity of the drug rather than longevity of symptoms[23]. Peer reviewed randomized control trials from Bangladesh support our finding of faster viral clearance in the ivermectin group, and in a small RCT from Spain, treatment with ivermectin showed a tendency toward faster viral clearance.[24, 25]

One may wonder about the public health benefit of treating mild patients since shortening the period of relatively mild symptoms may not merit a mass drug administration of ivermectin to the large population of non-hospitalized patients. However, inducing faster viral clearance and therefore reducing the time until the patient reaches a state of being non-infectious have an extremely important public health impact. Taking the two composites; Ct values above 30 and negative cultures, leads to almost 90% non-infectious status at day four (one day after ending treatment) among ivermectin users [] . From the public health point of view, it may shorten isolation time, which can serve as a major relief of the economic and social burden.

Our study has several limitations. First, the sample size was relatively small, and was designed to look for differences in viral load, but not for clinical deterioration and prevention of hospitalization. The second limitation was that drug therapy was not physically observed by investigators. Finally, our study was conducted among mild-non-hospitalized patients and therefore the results cannot be applied to a more severe or immune-suppressed populations.

The strength of our study was its double-blind structure with more substantial outcomes such as Ct values and culture viability where the laboratory personnel did not have any information concerning the patients' assignment.

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In conclusion, our study strongly supports the notion that ivermectin has anti-SARS-CoV-2 activity. If used at the early stage of disease onset, it may shorten the isolation time and reduce transmission. Further studies are needed to test its ability to prevent clinical deterioration for high-risk groups and to examine its potential as a prophylactic drug. Vaccines are now starting to become available, but it will take years before they are distributed worldwide. As this drug may also reduce mortality, urgent intervention with further well-designed studies are needed.

Conflict of Interest

None

Funding

None

Acknowledgement

We would like to thank Super-Pharm Professional for donating the drug and the placebo pills, Ms. Liraz Olmer for statistical analysis support, Ms. Rivka Goldis for administration aids, Dr. Emiliano Cohen for graph producing, and Mr. Nadav Cain for his logistic support. Finally we would like to thank the Dirctorate of Defense Research and Development (DDR&D) at Israel's Ministry of Defense and Home Front Command staff who helped us accessing the dedicated corona hotels, without their support the study could not been performed.

Access to data

All data supporting the results will be provided by the corresponding author upon publication.

Contibutions

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Eli Schwartz and Asaf Biber had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conceptualization: ES

Data curation: ES, AB, MM

Formal analysis: ES, AB, MM, OE

Investigation: AB, MM, GH, DL, LR, AS, IN, LK, OE, ES

Methodology: ES, AB, MM, OE

Supervision: ES, AB

Writing - original draft: ES, AB, DL

Writing - review & editing: all authors contributed, reviewed and approved the last draft.

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366 **Table 1: Baseline study population**

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	All (N=89)		Ivermectin group (N=47)		Placebo group (N=42)		P*
Male gender *n,(%)*	69	(78·4)	36	(78·3)	33	(78·6)	0·9718
Age *median (IQ range)*	35·0	(28·0-47·0)	36·0	(32·0-50·0)	33·5	(26·0-47·0)	0·2446
Weight *median (IQ range)*	79·0	70·0-86·0)	80·0	(70·0-90·0)	75·0	(67·0-85·0)	0·2545
Symptomatic *n, (%)*	72	(80·9)	37	(78·7)	35	(83·3)	0·5807
Days from symptoms onset** *median (IQ range)*	4·0	(3·0-5·0)	4·0	(3·0-5·0)	4·0	(3·0-5·0)	0·9170
Ct value on day 0 *median (IQ range)*	23·0	(20·0-28·0)	24·0	(21·0-28·0)	22·0	(19·0-27·0)	0·1030

368 *P value by Fisher exact test for categorical variables or by Kruskal–Wallis test for continuous variables

369 ** calculated for only for symptomatic patients

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372 **Table 2: The negative RT-PCR (Ct>30) test for SARS-CoV-2 results, ratio at days 4 to 10**

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	N	Ivermectin	Placebo	P value*	OR	95% CI	
A. Based on RT-PCR (Ct>30) test							
Day 4	50	15/28 (54%)	7/22 (32%)	0·12	2·47	0·77	7·92
Day 6	89	34/47 (72%)	21/42 (50%)	0·03	2·62	1·09	6·31
Day 8	89	39/47 (83%)	25/42 (59%)	0·01	3·32	1·25	8·82
Day 10	89	40/47 (85%)	29/42 (69%)	0·07	2·56	0·91	7·72
B. Based on RT-PCR (Ct>30) test together with non-viable cultures							
Day 4	50	24/28 (86%)	13/22 (59%)	0·03	4·1538	1·0688	16·1439
Day 6	89	44/47 (94%)	31/42 (74%)	0·01	5·2043	1·3400	20·2129
Day 8	89	45/47 (96%)	32/42 (76%)	0·01	7·0313	1·4419	34·2875
Day 10	89	45/47 (96%)	36/42 (86%)	0·14**	3·7500	0·7135	19·7078

374 *P value by Chi squre test , **P value by Fisher exact test

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376 **Table 3: Multivariable analysis for negative RT-PCR (Ct>30) test for SARS-CoV-2 results on day 6 and 8**

	Day 6				Day 8			
	Odds Ratio	95% Confidence Interval		P value	Odds Ratio	95% Confidence Interval		P value
Female	0·871	0·267	2·840	0·8186	0·495	0·138	1·772	0·2795
Age	0·983	0·949	1·017	0·3247	0·964	0·928	1·002	0·0597
Weight	1·004	0·974	1·035	0·7950	0·998	0·966	1·031	0·8927
Symptomatic	0·669	0·197	2·274	0·5194	0·609	0·139	2·665	0·5105
Ivermectin treatment	2·619	1·062	6·454	0·0365	3·871	1·357	11·041	0·0114

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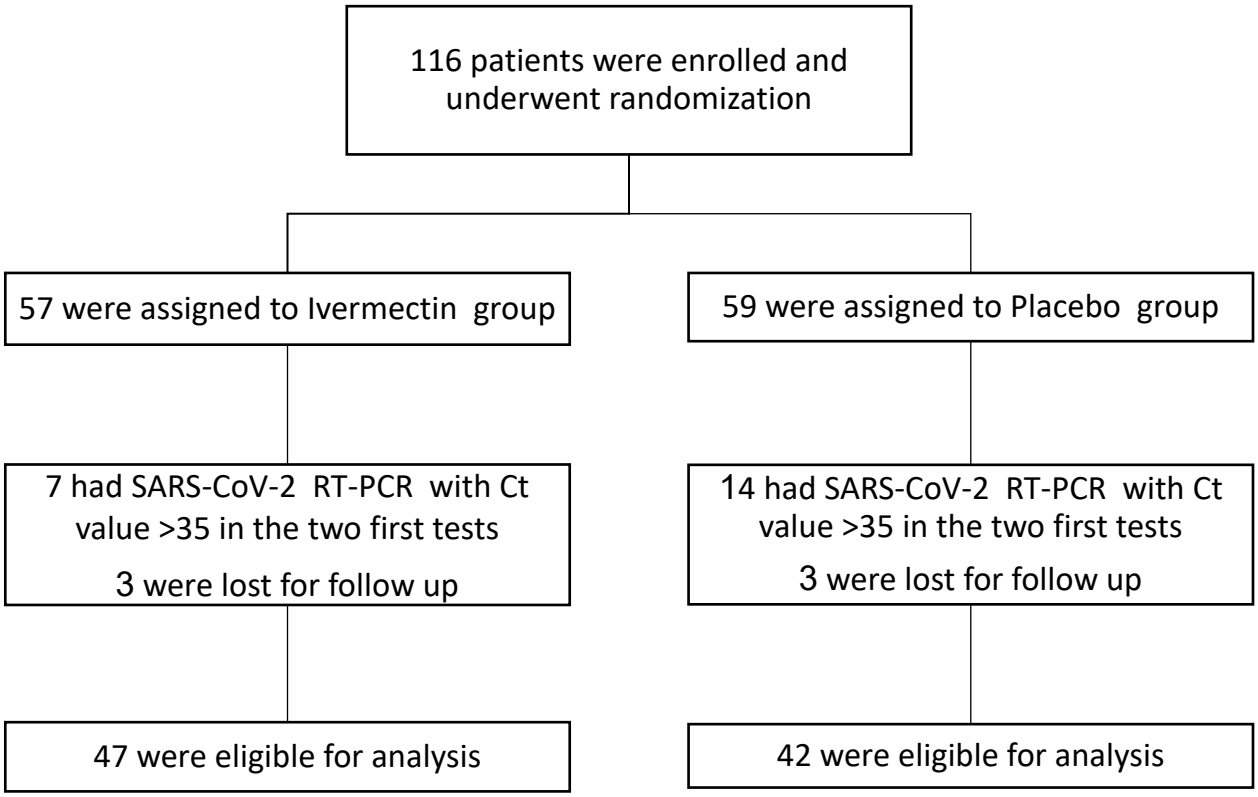
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390 **Figure 1:** Enrollment and patient flow

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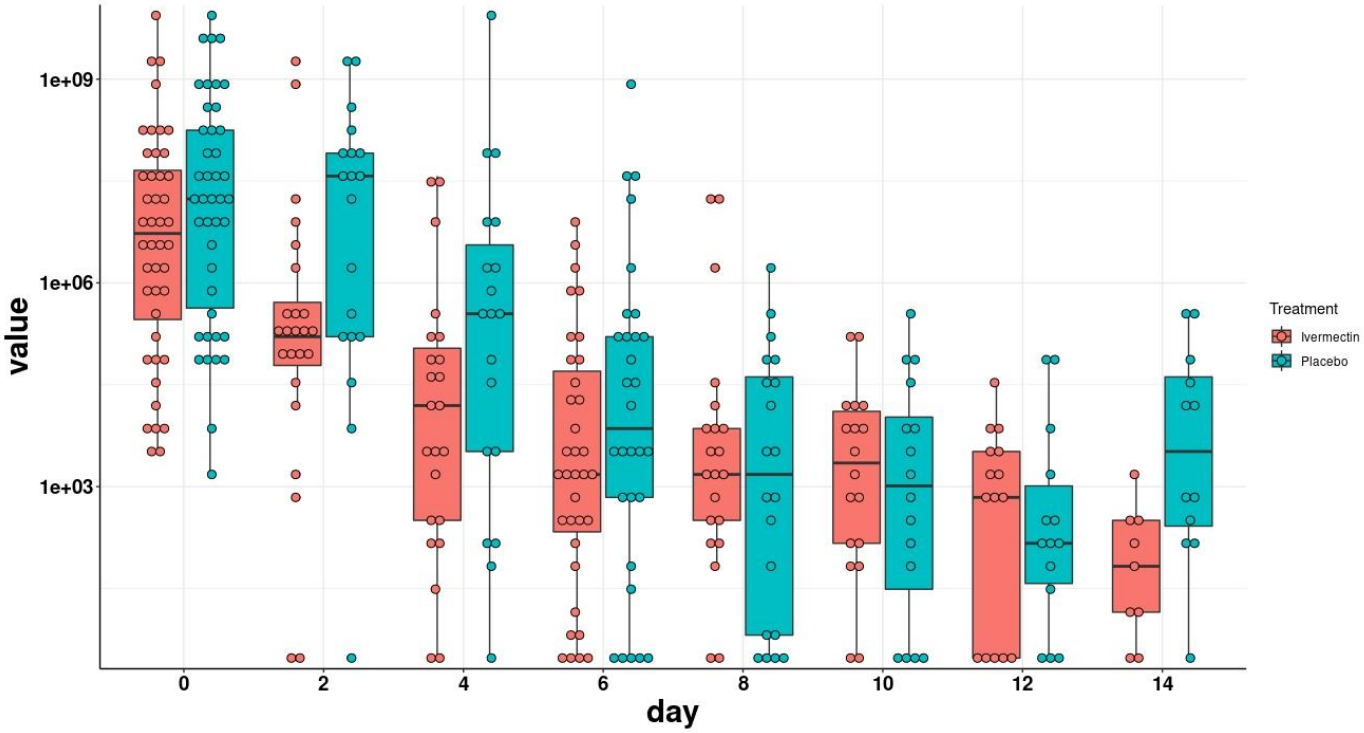


Figure 2: Viral load evolution by study arm. Viral load values were log-transformed. The boxes show the interquartile range. Dots represent each individual value.

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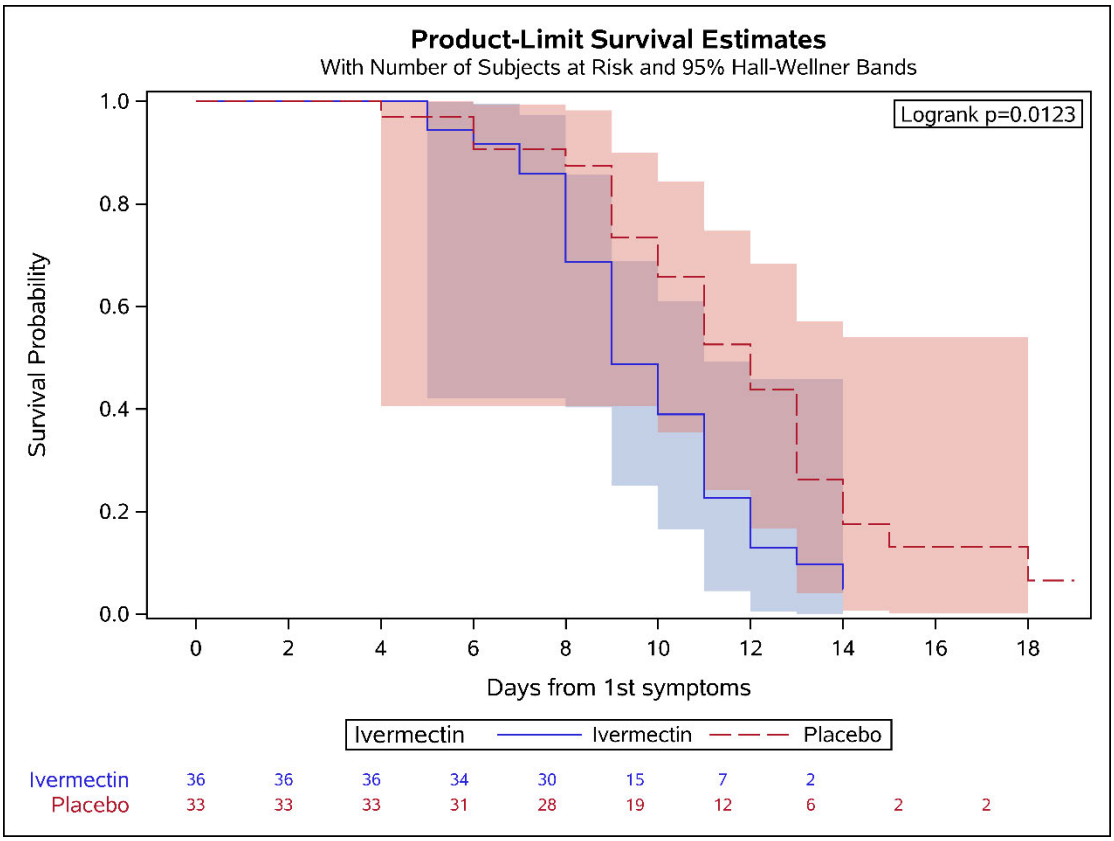


Figure 3: Kaplan-Meyer curve for time to negative (Ct <30) results for symptoms onset (calculation is done for symptomatic patients, N=69)

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International Immunopharmacology 18 (2014) 27–34

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Antibiotics regulate the immune response in both presence and absence of lipopolysaccharide through modulation of Toll-like receptors, cytokine production and phagocytosis *in vitro*

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ARTICLE INFO

Article history:
Received 20 June 2013
Received in revised form 18 October 2013
Accepted 19 October 2013
Available online 13 November 2013

Keywords:
Sepsis
Antibiotics
Toll-like receptors
Cytokines
Immunomodulation
Phagocytosis

ABSTRACT

The inflammatory response to pathogen-associated molecular patterns such as lipopolysaccharide (LPS) in sepsis is mediated *via* Toll-like receptors (TLRs). Since TLRs also trigger various immune functions, including phagocytosis, their modulation is a promising strategy in the treatment of sepsis. As antibiotics have immunomodulatory properties, this study examined the effect of commonly used classes of antibiotics on i) the expression of TLRs and cytokines and ii) the phagocytic activity under sepsis-like conditions *in vitro*. This was achieved by incubating THP-1 monocytes and peripheral blood mononuclear cells (PBMCs) obtained from patients after open-heart surgery with the addition of LPS and six key antibiotics (piperacillin, doxycycline, erythromycin, moxifloxacin or gentamicin). After 24 h, mRNA levels of both cytokines (IL-1β, IL-6) and TLRs (1, 2, 4, and 6) were monitored and phagocytosis was determined following coincubation with *Escherichia coli*. Each antibiotic differentially regulated the gene expression of the investigated TLRs and cytokines in monocytes. Erythromycin, moxifloxacin and doxycyclin displayed the strongest effects and changed mRNA-levels of the investigated genes up to 5.6-fold. Consistent with this, antibiotics and, in particular, moxifloxacin, regulated the TLR-and cytokine expression in activated PBMCs obtained from patients after open-heart surgery. Furthermore, piperacillin, doxycyclin and moxifloxacin inhibited the phagocytic activity of monocytes. Our results suggest that antibiotics regulate the immune response by modulating TLR- and cytokine expression as well as phagocytosis under septic conditions. Moxifloxacin, doxycycline and erythromycin were shown to possess the strongest immunomodulatory effects and these antibiotic classes should be considered for future immunomodulatory studies in sepsis.

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1. Introduction

Sepsis is one of the leading causes of death in critically ill patients and is associated with a hospital mortality of approximately 30% [1]. The clinical manifestation of sepsis is characterized by the cardinal signs of inflammation including tachycardia, leukocytosis, tachypnea, and pyrexia, which are collectively defined as systemic inflammatory response syndrome (SIRS). This systemic inflammatory response results from excessive stimulation of innate immune cells by exposure to different evolutionarily conserved molecular structures on infectious microorganisms, known as "pathogen-associated molecular patterns" (PAMPs)[2].

The recognition of PAMPs is mediated by pattern recognition receptors (PRR) including the Toll-like receptors (TLRs), the Nod-like receptors (NLRs), the C-type lectin receptors (CLRs) and the RIG-like receptors (RLRs) [3]. TLRs are the most widely studied PRRs and are considered to be the primary sensors of pathogens [4]. Lipopolysaccharide (LPS) released from gram-negative bacteria is recognized by TLR4 and comprise a major class of PAMP that causes sepsis through the induction of pro-inflammatory cytokines such as IL-1β and IL-6 [4,5]. Beside their role in the recognition of invading microbes, TLR-signaling further triggers the clearance of pathogens by phagocytosis, which plays a key role in the immune response during sepsis [6]. Due to the pivotal contribution of TLRs in sepsis, their modulation and signaling pathways became a research focus and it has been suggested that TLRs could be potential target molecules for the treatment of sepsis [7–9].

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http://dx.doi.org/10.1016/j.intimp.2013.10.025

FOI 26-3132 - Document 5

Although significant resources have been invested during the last decade in new treatments for sepsis, almost all have failed to improve outcomes and intervention with antibiotics remains one of the most effective strategies. Antibiotics have widely appreciated bacteriostatic or bactericidal properties and are used in the therapy of numerous infections, including those associated with sepsis. Interestingly, some antibiotics also directly modulate the immune system in addition to exerting their antimicrobial properties. Thereby, antibiotics interact with host immune cells and regulate their immune function, which has beneficial effects in various inflammatory conditions [10,11]. Previous studies have shown that antibiotics improve survival in animals challenged with LPS or multidrug-resistant pathogens, suggesting an immunomodulatory and non-antimicrobial effect of antibiotics under septic conditions [12–15]. Furthermore, clinical data showed for the first time that the immunomodulatory properties of macrolide antibiotics improve the outcome of septic patients [16].

Yet, little is known about the molecular and cellular mechanisms by which antibiotics modulate the immune system during sepsis. Thus, the aim of this study was to examine the effect of antibiotics on the expression of TLRs and sepsis-related cytokines under inflammatory conditions that mimic sepsis. We focused on classes of antibiotics that are commonly used in the treatment of sepsis and investigated their effects on LPS-activated human immune cells, including leukocytes isolated from patients with a systemic inflammatory response after open-heart surgery. These research findings clearly indicate that the classes of chinolones (moxifloxacin), tetracyclines (doxycycline) and macrolides (erythromycin) regulate the expression of TLRs and cytokines in septic conditions. Thus, use of these antibiotics can be one useful clinical treatment for septic patients.

2. Material and methods

2.1. Reagents and THP-1 cells

LPS from *Escherichia coli* (serotype 055:B5) was purchased from Sigma-Aldrich (Taufkirchen, Germany) and antibiotics were obtained from the manufacturer: gentamicin (GMC), piperacillin (PIP) and doxycycline (DXC) from Ratiopharm (Ulm, Germany); erythromycin (ERY) from Amdipharm (Dublin, Ireland) and moxifloxacin (MXF) from Bayer (Leverkusen, Germany). Human monocytic THP-1 cells (ATCC TIB-202) [17] were grown in suspension in RPMI1640 (PAA, Coelbe, Germany) containing 10% FCS (Biochrom, Berlin, Germany), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany), 100 mg/mL penicillin, 100 mg/mL streptomycin (both from Invitrogen, Frankfurt, Germany) and were maintained at 37 °C in a humidified incubator containing 5% CO_2. The cells were cultured in antibiotic- and FCS-free suspension for 48 h before experimental use to avoid any confounding stimulation that might result from antibiotics or endotoxins present in the cell culture medium. The viability of monocytes was determined by the tryptan blue staining method. Cells with viability greater than 90% were used for the experiments.

2.2. Patients and healthy volunteers

With the approval of the Ethics Committee of the Rheinische Friedrich-Wilhelms-University Medical School in Bonn (number of approval: 125/08), and following written informed consent, we recruited six healthy, non-smoking male volunteers and eight patients scheduled to undergo cardiopulmonary bypass (CPB) for routine elective coronary artery grafting. We excluded patients with acute or chronic infection, immunocompromised patients, patients with autoimmune disorders, or those taking immunosuppressive or anti-infective drugs in the preceding five days. All patients underwent a routine procedure with median sternotomy and use of CPB. Anesthesia was induced with etomidate, sufentanil and cisatracurium and was maintained using isoflurane and sufentanil. Thereafter, the patients received 1.5 g cefuroxime as perioperative antibiotic prophylaxis. The CPB technique included non-pulsatile flow with moderate hypothermia (32 °C–34 °C) and the CPB pump was primed with a standard electrolyte solution containing 10,000 IU heparin. Additional heparin was given if the activated clotting time failed to reach at least 480 s. Cardioplegia was induced by application of Calafiore warm blood-cardioplegic solution. Weaning from CPB was achieved after rewarming and inotropic support with epinephrine (0.025–0.15 μg/kg/min) in all patients and additional norepinephrine (0.025–0.15 μg/kg/min) as necessary. After CPB, heparin was neutralized with protamine sulfate until the preoperative activated clotting time was achieved. Postoperative fluid management consisted of isotonic-balanced electrolyte solution (30–40 ml/kg/day) for basic replacement and gelatin, fresh frozen plasma, or packed red cells according to hemodynamic status and laboratory parameters. Postoperative antibiotic prophylaxis was performed with 3 × 1.5 g cefuroxime every 8 h after admission on ICU.

2.3. Blood collection and preparation of peripheral blood mononuclear cells

Blood samples obtained from healthy volunteers were collected by peripheral vein puncture. Patient blood samples were taken through an indwelling central venous catheter before induction of anesthesia, 30 min and 24 h after end of surgery. In general, blood collection was performed before routinely planned administration of antibiotic prophylaxis. Monovette blood collecting system (Sarstedt, Nuembrecht, Germany), containing EDTA as anticoagulant, was used. Peripheral blood mononuclear cells (PBMCs) were immediately isolated from blood samples by sucrose gradient (PANcoll, PAN-biotech, Aidenbach, Germany) as described elsewhere [18]. After washing, PBMCs were cultured as described above.

2.4. In vitro stimulation experiments

THP-1 cells and PBMCs at a concentration of 1×10^6 cells/mL in 6-well plates (Cellstar, Greiner bio-one, Frickenhausen, Germany) were separately incubated in the presence of either LPS (10 μg/ml), GMC (5 μg/ml), ERY (2 μg/ml), MXF (20 μg/ml), DXC (8 μg/ml), PIP (100 μg/ml) or a combination of LPS with each antibiotic. The cells were incubated at 37 °C and were removed from culture plates after 24 h for isolation of RNA.

2.5. Isolation of RNA and Real-time PCR

Total RNA was isolated from the THP-1 cells and PBMCs using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was generated from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) as recommended by the manufacturer. Cycling conditions were 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s. Real-time PCR was carried out on the 7900HT Fast Real-Time PCR system from Applied Biosystems using TaqMan Gene Expression Assays primer/probe sets (Applied Biosystems) and the standard thermal-cycling conditions for relative quantification designed by the manufacturer. Quantification of the PCR signals for each sample was performed by comparing the cycle threshold values in duplicate for the gene of interest with the cycle threshold values for the GAPDH housekeeping gene. The mean relative mRNA expression was determined by using the SDS software V2.2 and the $2^{-\Delta\Delta CT}$ method [19].

2.6. Phagocytosis assay

The phagocytic activity of THP-1 cells was examined by measuring the uptake of pHrodo *E. coli* BioParticles (Invitrogen) as described by Wan et al. [20]. To prepare BioParticles, 2 ml of PBS were used for reconstitution of the BioParticles. THP-1 cells (1×10^6 cells/ml) were incubated with antibiotics in the presence or absence of LPS as

FOI 26-3132 - Document 5

described above for 24 h, followed by an additional incubation with pHrodo *E. coli* BioParticles for 2 h. DNA staining for viability measurement was performed just prior to flow cytometry analysis with 2 μg/ml Hoechst 33342 (Invitrogen). Flow cytometry was conducted using a Canto III Flow Cytometer System (BD Biosciences, Heidelberg, Germany) and FlowJo Analysis Software (Treestar, Ashland, OR, USA). Phagocytic activity was expressed as the percentage of cells that performed phagocytosis compared to the total number of viable monocytes.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Mann–Whitney *U* test was used to examine differences in phagocytic assay and in gene expression of both THP-1 cells and PBMCs from healthy volunteers. Data obtained from CPB surgery patients were analyzed by paired *t*-test. Values of $p < 0.05$ were considered significant. All data are expressed as mean ± SEM unless otherwise noted.

3. Results

3.1. Antibiotics differentially modulate the gene expression of TLRs and cytokines in monocytes

To determine a putative immunomodulatory effect of antibiotics in sepsis, we initially incubated human THP-1 monocytes with clinically relevant concentrations of commonly used classes of antibiotics, namely PIP (beta-lactam), DXC (tetracycline), ERY (macrolide), MXF (quinolone), and GMC (aminoglycoside) in the presence or absence of LPS. The expression of genes encoding IL-1β, IL-6, TLR1, TLR2, TLR4, and TLR6 was monitored by RT-PCR after 24 h. Tested antibiotics showed differential regulatory effects on gene expression of the investigated cytokines and TLR-subtypes (Fig. 1). The magnitude of this regulation differed between the various classes of antibiotics. Whereas ERY displayed strong immunomodulatory properties in terms of increasing the gene expression up to 5.6-fold (TLR4; Fig. 1E; $p < 0.05$) in LPS-stimulated cells, PIP only altered the mRNA levels up to 2.3-fold (TLR1; Fig. 1C; $p < 0.05$) compared to LPS-activated controls. In detail, PIP increased the expression of TLR1, TLR2, TLR4 and TLR6 by ≈2-fold when compared to unstimulated controls (Fig. 1C–F; $p < 0.05$). In co-stimulation with LPS, the number of genes regulated by the presence of PIP also included IL-1β and IL-6 (Fig. 1A,B; $p < 0.05$). In contrast, cells incubated with either DXC or GMC alone exhibited significantly reduced mRNA levels of IL-1β (DXC and GMC; Fig. 1A; $p < 0.05$), TLR1 (DXC, Fig. 1C; $p < 0.05$), TLR4 (GMC, Fig. 1E, $p < 0.05$) and TLR6 (DXC and GMC, Fig. 1F; $p < 0.05$). Furthermore, co-incubation of monocytes with LPS led to a significant down-regulation of IL-6 by both DXC and GMC compared to their LPS-stimulated controls (Fig. 1B; $p < 0.05$). The incubation of monocytes with either ERY or MXF resulted in differential regulation of several genes: THP-1 cells cultured in the presence of ERY alone showed significantly higher mRNA levels of IL-1β ($p < 0.05$), whereas ERY in combination with LPS reduced mRNA levels of IL-1β when compared to their respective controls (Fig. 1B; $p < 0.05$). THP-1 monocytes stimulated with ERY ± LPS exhibited significantly increased mRNA levels of TLR1, TLR2, TLR4 and TLR6 (Fig. 1C–F; $p < 0.05$). The activation status of monocytes also influenced the regulation of TLR4 gene expression by MXF. In LPS-activated cells, MXF significantly increased the TLR4 expression 2-fold, while MXF reduced the TLR4 mRNA level by more than 50% in the absence of endotoxin (Fig. 1E; $p < 0.05$). Furthermore, MXF significantly reduced IL-6 and TLR6 expression when cultured in the absence of LPS (Fig. 1B,F; $p < 0.05$) and reduced both IL-1β and IL-6 mRNA levels in LPS-activated cells (Fig. 1A,B; $p < 0.05$).

3.2. Antibiotics differentially modulate the gene expression of TLRs and cytokines in PBMCs

The immunomodulatory effects of antibiotics on a mixed immune cell population were studied in PBMCs obtained from healthy human donors and were stimulated under the same conditions as described above. Consistent with the results from THP-1 monocytes, PIP increased

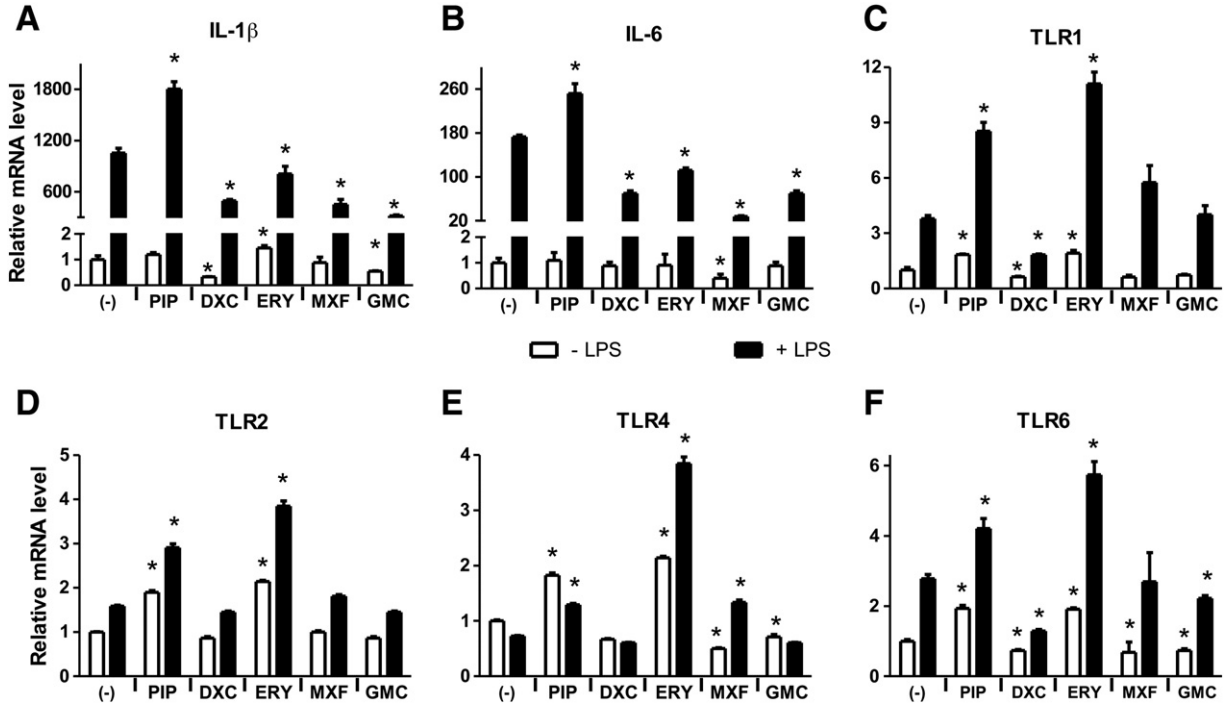


Fig. 1. Effect of antibiotics on cytokines and TLR expression in endotoxin-activated THP-1 monocytes. THP-1 monocytes were incubated with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence or absence of LPS for 24 h. Quantitative Real-Time-PCR (qRT-PCR) analysis was performed to determine the expression levels of genes encoding IL-1β (A), IL-6 (B), TLR1 (C), TLR2 (D), TLR4 (E) and TLR6 (F) after normalization to GAPDH. Each bar represents the fold-change in mRNA expression comparing unstimulated control with antibiotic-incubated cells or LPS-stimulated control with LPS plus antibiotic-stimulated cells, respectively. Data are expressed as mean ± SEM; *, $p < 0.05$.

FOI 26-3132 - Document 5

the TLR2 expression, whereas DXC, MXF and GMC reduced the mRNA levels of TLR4 and/or TLR6 in PBMCs compared to their unstimulated controls (Fig. 2D–E; $p < 0.05$). Furthermore, down-regulation of TLR6 mRNA expression by both DXC and GMC was also found in LPS-activated PBMCs (Fig. 2F; $p < 0.05$). In contrast to the observed immunosuppressive effects of antibiotics on cytokine expression in the THP-1 cell line, PBMC cultured with DXC, MXF or GMC alone and GMC in combination with LPS exhibited significantly higher mRNA levels of IL-1β as compared to the respective controls (Fig. 2A, $p < 0.05$). Furthermore, PBMCs cultured with MXF alone revealed significantly increased mRNA level of IL-6 compared to unstimulated cells (Fig. 2B; $p < 0.05$).

3.3. Antibiotics differentially modulate the gene expression of TLRs and cytokines in PBMCs from patients with a systemic inflammatory response

We next studied the immunomodulatory effects of antibiotics on immune cells from patients with a systemic inflammatory response after open-heart surgery to mimic conditions found in sepsis. Therefore, PBMCs were isolated from 8 patients undergoing CPB-surgery before induction of anesthesia as well as 30 min and 24 h after surgery, followed by incubation with antibiotics and LPS as described above. The operation was uncomplicated in all of the patients and their demographic data as well as clinical profile are presented in Table 1. All patients were broadly immunostimulated, which was represented by a significant increase in the number of leukocytes after surgery (Table 2; $p < 0.05$). Yet, further parameters to diagnose a SIRS after surgery could not be evaluated as these were manipulated by routinely performed treatments such as cardiac pacing and mechanical ventilation. Blood from two patients could not be collected at 24 h after surgery due to their unstable condition. The PBMCs responded to the surgery by down-regulating mRNA levels of TLR2 after 30 min and both IL-1β and IL-6 after 24 h compared to preoperative collected cells (Figs. 3 and 4B; $p < 0.05$). In LPS-activated PBMCs, this effect was extended by up-regulation of IL-1β, IL-6, TLR1, TLR4 and TLR6 in cells obtained after 30 min and down-regulation of TLR2 in cells isolated 24 h after surgery compared to preoperative controls (Figs. 3 and 4; $p < 0.05$). Those effects were partially modulated by antibiotics, suggesting an immunomodulatory effect of antibiotics on either one or both studied time points (Figs. 3 and 4).

Therefore, we analyzed the effect of antibiotics on gene expression within each time point and found that in pre-operatively isolated PBMCs DXC increased the expression both IL-1β and IL-6, while PIP, DXC and MXF significantly increased the mRNA levels of TLR2 and reduced the TLR1 gene expression compared to unstimulated cells (Figs. 3 and 4A,B; $p < 0.05$). Furthermore, PIP significantly increased the

Table 1
Demographic data and perioperative characteristics of patients undergoing CPB surgery.

Patient characteristics (n = 8)	
Age (Years)	72.5 ± 8.3
Weight (kg)	83.0 ± 12.8
Height (cm)	171.9 ± 8.5
Pre-operative ejection fraction (%)	61.0 ± 13.2
Perioperative Data	
Duration of surgery (min)	258.6 ± 75.2
CPB time (min)	66.3 ± 12.4
ACC (min)	16.3 ± 23.5
Intubation time (h)	27.5 ± 9.5
Postoperative blood loss (ml)	1247.5 ± 466.4
Length of stay on ICU (h)	47.1 ± 26.7
Intraoperative red blood cell transfusion (n)	5/8
Postoperative red blood cell transfusion (n)	7/8

Values are presented as mean ± SD except for the number of transfused red blood cells. CPB indicates cardiopulmonary bypass; ACC, aortic cross-clamping; ICU, intensive care unit.

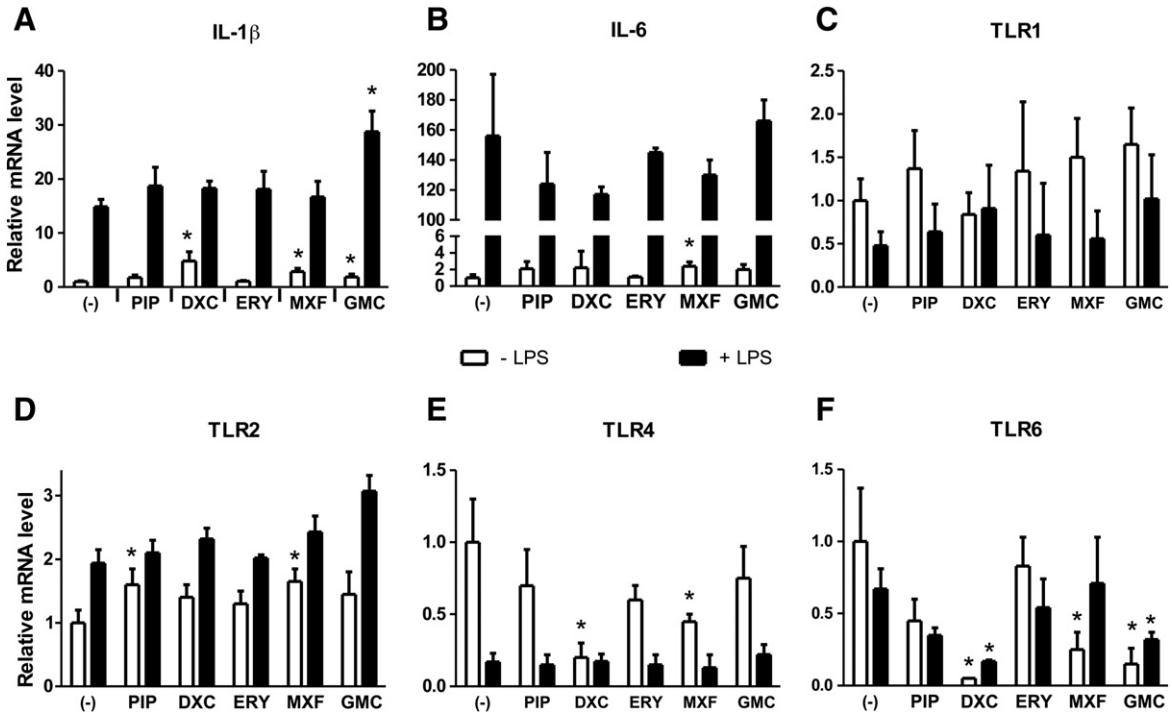


Fig. 2. Effect of antibiotics on cytokine and TLR-expression in endotoxin-activated PBMCs from healthy human donors *ex vivo*. Freshly isolated PBMC from healthy human donors were cultured with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence or absence of LPS for 24 h and mRNA expression of IL-1β (A), IL-6 (B), TLR1 (C), TLR2 (D), TLR4 (E) and TLR6 (F) was measured by quantitative Real-Time PCR. Data represent the fold-change in mRNA expression comparing unstimulated control to antibiotic-incubated cells or LPS-stimulated control to LPS plus antibiotic-stimulated cells, respectively. Data are expressed as mean ± SEM of 6 individual donors in each group; *, $p < 0.05$.

FOI 26-3132 - Document 5

Table 2
Perioperative laboratory data and vital parameters of patients undergoing coronary artery bypass surgery.

Variable	Preoperative	End of CPB (n = 8)	24 h postop (n = 6)
Laboratory data			
Leucocyte count ($\times 10^9$/L)	7.0 ± 1.2	13.6 ± 2.1*	8.0 ± 2.7
Hemoglobin (mg/dL)	13.1 ± 1.2	10.5 ± 0.8	10.5 ± 0.8
Platelet count ($\times 10^9$/L)	224 ± 70	118 ± 65	117 ± 41
Vital Parameters			
Heart rate (/min)	61 ± 18	96 ± 6[a]	95 ± 13[a]
Mean arterial pressure (mmHg)	68 ± 6.7	83 ± 8	
73 ± 13			
Temperature (°C)	36.3 ± 0.3	36.5 ± 0.7	37.8 ± 0.4

Values are presented as mean ± SD.
* $p < 0.05$ *vs.* preoperative data.
[a] Patients received cardiac pacing.

expression of the gene encoding TLR6 compared to unstimulated pre-operatively collected PBMCs (Fig. 4D; $p < 0.05$). In LPS-activated PBMCs, only ERY reduced the mRNA-level of TLR1 whereas GMC increased IL-1β expression (Figs. 3A and 4A; $p < 0.05$). When PBMCs were stimulated *in vivo* by surgery and collected 30 min after surgery, PIP in co-stimulation with LPS significantly reduced their mRNA expression of TLR1 and TLR4 while TLR2 expression was increased compared to LPS-activated controls (Fig. 4; $p < 0.05$). The down-regulation of IL-1β and TLR2 by ERY also required the presence of LPS, whereas MXF increased both IL-1β and TLR2 expression and decreased TLR4 and TLR6 expression in the absence of LPS. The presence of LPS was also important for the regulatory activity of DXC: DXC alone significantly reduced TLR1 while DXC in combination with LPS increased TLR1 expression significantly compared to their respective controls (Fig. 4A; $p < 0.05$). When PBMCs were obtained from patients 24 h after CPB surgery, only MXF significantly reduced the mRNA level of TLR4 while no effect by other antibiotics was observed (Fig. 4C; $p < 0.05$).

3.4. Effect of antibiotics on bacterial phagocytosis

After monitoring an effect of antibiotics on the expression of TLRs, which play a critical role in regulating the phagocytosis of bacteria [6], we examined whether antibiotics may affect the phagocytosis of immune cells. Therefore, THP-1 monocytes were stimulated with antibiotics and LPS as described above, followed by incubation with heat-killed *E. coli*. As determined by flow cytometry, the inclusion of PIP, DXC, or MXF in combination with LPS during culturing decreased the percentage of phagocytizing cells (Fig. 5; $p < 0.05$ and $p < 0.01$, respectively). This effect was LPS-dependent as incubation with antibiotics alone had no effect on the phagocytic activity (data not shown).

4. Discussion

The excessive inflammatory response to microbial pathogens in sepsis is mediated *via* TLRs and their modulation might improve the outcome of septic patients by limiting the pathological inflammation [21]. Increasing evidence suggests that the immunomodulatory properties of antibiotics may have beneficial effects in sepsis [12–15]. Yet, the underlying molecular and cellular mechanisms including the effect of antibiotics on TLR expression remains unclear. The current study showed immunomodulatory properties of various classes of antibiotics on the expression of TLRs and sepsis-associated cytokines. Those effects were particularly found in immune cells that were activated by the sepsis mediator LPS and during a systemic inflammatory response after surgical trauma.

Innate immune cells, such as monocytes, play a central role in the pathogenesis of sepsis and are rapidly stimulated by various PAMPs, in particular LPS, originating from invading pathogens [2]. LPS triggers a

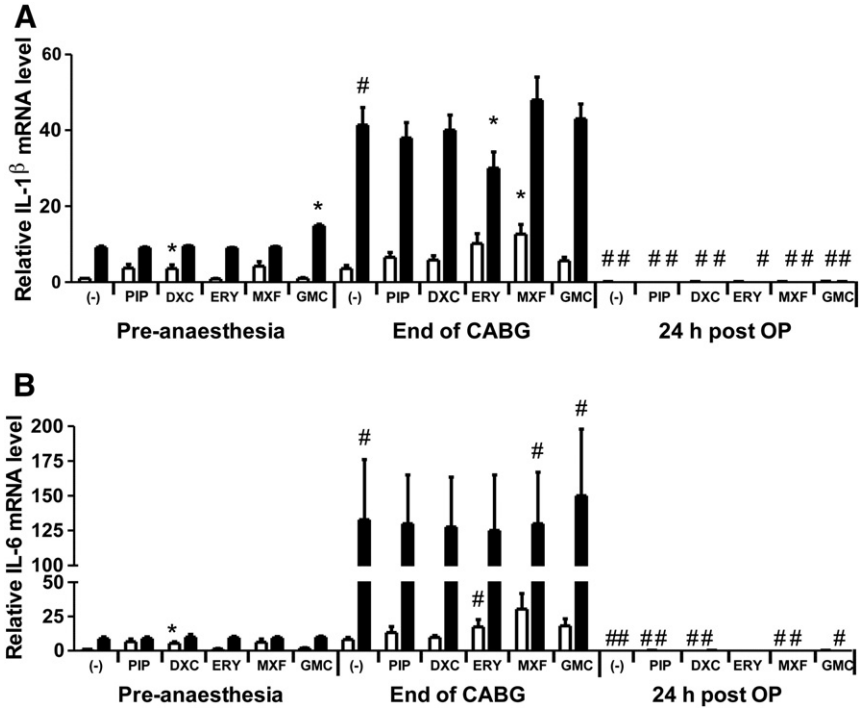


Fig. 3. Effect of antibiotics on cytokine expression in endotoxin-activated PBMCs before, 30 min and 24 h after CPB surgery. PBMCs from 8 patients undergoing CPB surgery were obtained before anesthesia, 30 min (End of CPB) and 24 h after operation (post op). Cells were cultured with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence (solid bar) or absence (open bar) of LPS for 24 h and mRNA expression of IL-1β (A) and IL-6 (B) was measured by quantitative Real-Time PCR. Data are expressed as mean ± SEM and normalized unstimulated cells, which were collected at pre-operative time point. # indicates $p < 0.05$ compared to pre-anesthesia; in intergroup comparisons at the various time points * indicated $p < 0.05$.

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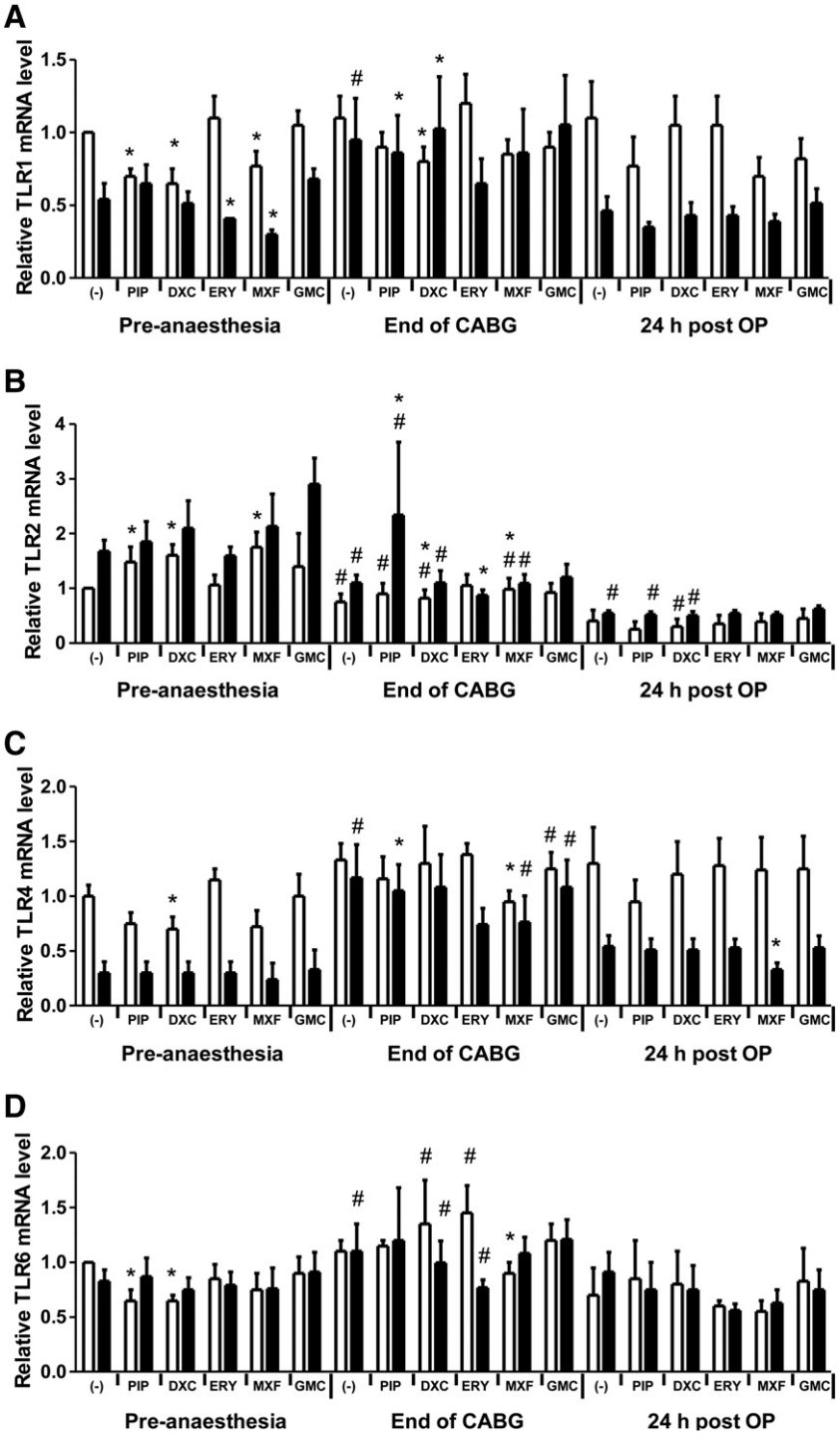


Fig. 4. Effect of antibiotics on TLR expression in endotoxin-activated PBMCs before, 30 min and 24 h after CPB surgery. PBMCs from 8 patients undergoing CPB surgery were obtained before anesthesia, 30 min (End of CPB) and 24 h after operation (post op). Cells were cultured with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence (solid bar) or absence (open bar) of LPS for 24 h and mRNA expression of TLR1 (A), TLR2 (B), TLR4 (C), and TLR6 (D) was measured by quantitative Real-Time PCR. Data are expressed as mean ± SEM and normalized to unstimulated cells, which were collected at pre-operative time point. # indicates $p < 0.05$ compared to pre-anesthesia; in intergroup comparisons at the various time points * indicated $p < 0.05$.

pro-inflammatory response, which is characterized by a production of inflammatory cytokines such as IL-1β and IL-6, *via* TLR4 [4]. Furthermore, LPS-activation changes the expression of TLRs in immune cells and leads to a differential response to subsequent PAMP exposure, which can result in an excessive pro-inflammatory response or immunocompromised status of a septic patient [22]. Consistent with previous reports, we found that tetracyclines (DXC), macrolides (ERY) and fluoroquinolones (MXF) had anti-inflammatory properties, which manifest by inhibiting the expression of inflammatory cytokines in response to LPS [13,23–25]. At the same time, beta-lactams (PIP) showed pro-inflammatory effects by increasing mRNA levels of IL-1β and IL-6 [26]. Furthermore, the aminoglycoside GMC also reduced the mRNA levels of IL-1β and IL-6 in monocytes which is in agreement with the effects of GMC on renal tubule cells [27]. We demonstrated regulatory properties for all tested classes of antibiotics on TLR expression to a various extent. These effects ranged from modest effects found in GMC-

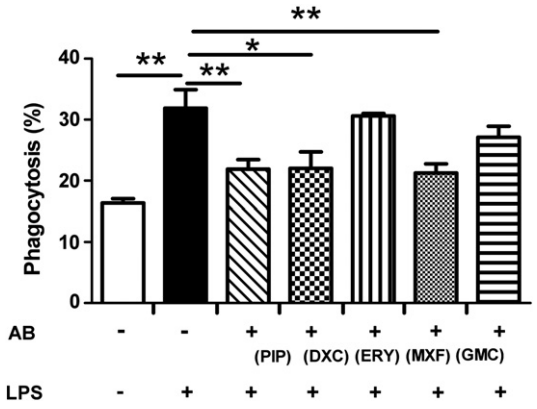


Fig. 5. Effect of antibiotics on phagocytosis of heat-inactivated *E. coli* by THP-1 cells. THP-1 cells were incubated with piperacillin (PIP), doxycycline (DXC), erythromycin (ERY), moxifloxacin (MXF) or gentamicin (GMC) in the presence of LPS for 24 h and were incubated thereafter for 2 h with heat-killed rhodamine-conjugated *E. coli*. DNA staining for viability measurement was performed just prior to flow cytometry analysis with Hoechst 33342. Data show percentage of monocytes positive for phagocytosis determined by flow cytometry and are expressed as mean ± SEM; *, $p < 0.05$; **, $p < 0.01$.

incubated cells to a very significant modulation of all investigated TLR-subtypes by ERY. Interestingly, the observed effects of antibiotics on monocytes did not correspond to the results observed in PBMCs. For example, MXF increased the expression of IL-1β and IL-6 in PBMCs whereas the same genes were down-regulated by MXF in monocytes. This observation that antibiotics have different immunomodulatory effects on monocytes and heterogeneous leukocyte populations such as PBMCs, which consist of lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells, has been previously described. Bailly et al. demonstrated that the quinolone ciprofloxacin suppressed the pro-inflammatory cytokine response to endotoxin in monocytes while the treatment of a mixed cell population with ciprofloxacin increased their expression [28,29]. These results suggest that cellular interactions could modify monocytic TLR- and cytokine expression to a modulation by antibiotics.

Although the current data do not provide evidence for the immunomodulation by antibiotics at protein levels, several studies showed that the regulation of the investigated mRNA for TLRs and cytokines is correlated with the change in protein expression [30–32]. Thus, those findings suggest that antibiotics modulate the responsiveness of immune cells to LPS, which might help to understand why antibiotics such as the macrolide clarithromycin have beneficial immunomodulatory effects during sepsis [16].

The molecular mechanisms that underlie the immunomodulatory effects of antibiotics are so far poorly understood. To the authors' knowledge, no previous study has revealed the mechanisms causing the observed effects of PIP and GMC on the immune response. Yet, DXC has been identified to inhibit LPS-induced NO production by immune cells *in vitro* [23]. DXC may also be responsible for the reduction of IL-1β and IL-6 expression. In addition, tetracyclines such as DXC possess strong chelating abilities, which have been associated with immunomodulatory effects by inhibiting protein kinase C (PKC) [33,34]. Since it has been shown that PKC inhibitors reduce LPS-stimulated cytokine secretion [35], the chelating abilities of DXC might also reduce the IL-1β and IL-6 expression through PKC inhibition. The macrolid ERY was found to block the activation of transcription factors NF-κB and IRF3 [24,36], thereby reducing the production of both pro- and anti-inflammatory cytokines. The inhibition of signaling pathways by ERY depends on which PAMP the immune cell is activated by. While IRF3 is inhibited by ERY after LPS stimulation, no effect of ERY on NF-κB, which is critical for the expression of IL1-β and IL-6, has been detected [24]. Thus, the observed effect in the current study might be due to an unknown molecular mechanism. In contrast to ERY, MXF also inhibits the activation of NF-κB by inhibition of I-κB degradation after LPS stimulation [37]. Furthermore, MXF also blocks the activation of mitogen-activated protein kinases, which leads to a suppression of pro-inflammatory cytokines. These inhibitory effects of MXF on the TLR4/LPS-signaling pathway could explain its anti-inflammatory properties including the reduction of the pro-inflammatory cytokines IL-1β and IL-6.

For the first time, we found that the phagocytic activity of LPS-stimulated THP-1 cells toward *E. coli* was inhibited by PIP, DXC and MXF regardless of their effects on TLR expression. The inhibiting properties of MXF on the phagocytic activity have been previously described for the internalization of the gram-positive bacterium *Staphylococcus aureus* [38]. In contrast, another report showed that PIP enhances the phagocytic activity of *E. coli* by murine macrophages [39]. However, the comparison of studies on the phagocytic activity, which focus on murine cells is limited as murine and human immune cells differentially express phagocytic receptors after LPS stimulation [40]. A TLR-independent modulation of these receptors, such as scavenger receptors (SR) or CLRs, might explain inhibitory effects of PIP, DXC and MXF. This constellation was previously described for the effect of the immunomodulatory agent resveratrol on the phagocytosis of bacteria by monocytes [41]. There, resveratrol inhibited phagocytic activity of THP-1 cells by reducing SR and CTRs expression regardless of TLR-signaling. Furthermore, it has been demonstrated that different patterns of cytokines can also modulate the activity of human phagocytes [42]. Since the effect of antibiotics on cytokine production is presumably not limited to IL-1β and IL-6, the generation of a distinct pattern of cytokines by PIP, DXC and MXF could be another reason for the observed effects of antibiotics on phagocytosis.

The examination of direct immunomodulatory effects of antibiotics on immune cells from septic patients is limited due to preexisting combined antibiotic treatment. Given that sepsis is considered a systemic inflammatory response to a suspected or confirmed infection, we examined the effect of antibiotics on immune cells from patients after open-heart surgery. This population exhibits a frequent and robust inflammatory response, which is caused by surgical trauma, CPB, reperfusion injury and endotoxemia [43,44].

As previously reported, the peak of the pro-inflammatory cytokine expression occurred immediately after surgery [45,46]. Furthermore, we observed increased cytokine production after LPS stimulation in immune cells isolated at the end of the operation, whereas no cytokine response to LPS was detectable in cells collected 24 h post-operation. This indicates an early increase in endotoxin sensitivity and late endotoxin tolerance of the collected immune cells. Consistent with this, PBMCs obtained directly after surgery showed an up-regulation of TLR4 expression that is also observed in septic patients [30]. The immunomodulatory effect of antibiotics on PBMCs collected from patients before surgery was in accordance with the effects observed in PBMCs from healthy donors. We further found that all antibiotics (with the exception of GMC) showed to a various extent effects on the expression of cytokines and/or TLRs in immune cells that were *in vivo* activated though CPB surgery. Here, MXF showed the most immunomodulatory effects. Furthermore, the up-regulation of TLR2 expression and the reduction of TLR4 mRNA levels by MXF were also observed in all investigated cell populations including THP-1 monocytes.

The modulation of the immune system *via* TLRs is a promising strategy in the treatment of sepsis [7–9]. Current findings suggest that several classes of antibiotics, which are commonly used in the treatment of sepsis, regulate the immune system in sepsis-like conditions through modulation of the expression of TLRs and pro-inflammatory cytokines as well as the phagocytotic activity. We found that these effects varied in the antibiotics belonging to different classes and thus identified quinolones, tetracycline and macrolides as classes with the highest potential to modulate the TLR expression in septic conditions. Thus, these antibiotics should be considered for future immunomodulatory studies in sepsis.

FOI 26-3132 - Document 5

Acknowledgments

We acknowledge the editorial assistance of the NIH Fellows Editorial Board.

Conflict of interest statement

The authors have no conflicts of interest.

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FOI 26-3132 - Document 5
OPINION
published: 08 May 2020
doi: 10.3389/fmed.2020.00200

Doxycycline: From Ocular Rosacea to COVID-19 Anosmia. New Insight Into the Coronavirus Outbreak

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Keywords: COVID-19, doxycycline, coronavirus (CoV), SARS – CoV, anosmia

INTRODUCTION

Coronavirus Disease 19 (COVID-19) caused by Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) it usually manifests with respiratory symptoms (1).

Similarly, to other human respiratory Coronaviruses (HCoV), it seems to have a neuroinvasive and neurotropic activity (1, 2). In the retrospective case series study conducted by Mao et al. three categories of neurological symptoms COVID19-related included central nervous system (CNS) manifestations, peripheral nervous system (PNS) symptoms and musculoskeletal disorders (2).

Hyposmia has been reported as a possible peripheral nervous system (PNS) symptom caused by COVID-19 infection (2).

In our experience, the smell alteration (hyposmia, anosmia) seems to be one of the first manifestations of COVID-19 disease, with or without the loss of taste (dysgeusia). Sometimes it remains the only symptom; more often, it comes with fatigue, fever, and cough.

We provide a commentary on how COVID-19 could affect the sense of smell and the reason why doxycycline (Dox) could play a role in its recover.

ANOSMIA

The three leading causes of loss of smell reported in the literature are head trauma, chronic sinonasal inflammation and upper respiratory tract viral infections (3, 4). Anosmia is one between numerous olfactory disorders, but its mechanism is not clearly defined (3).

Post viral temporary chemosensory dysfunction after a common cold is widely reported (3, 5, 6).

The swelling of the mucosa in the olfactory cleft it seems to be cause of the transient olfactory and taste loss typically reported during the common cold. It usually leads to a conductive post-viral loss of smell, and it usually appears 3 months after the upper respiratory tract infection (3).

The olfactory neuroepithelium represents an important immunological barrier within the nasal cavity exposed to the external environment, and thus it is subject to both exogenous insults and endogenous host defense responses (7).

In the pathogenesis of chronic rhinosinusitis (CRS)-associated olfactory loss, interferon (IFN)γ-signaling pathways may play a pivotal role in orchestrating immune system function. They are able to modulate inflammatory response and pattern-recognition receptors expressed by the innate immune system during infection (7). Therefore, they support an inflammatory process underlying the olfactory impairment CRS-linked (8). As evidence of this (CRS)-associated olfactory dysfunction is relatively rapidly reversed with systemic corticosteroids (9).

On the other side, some post-viral sense of smell impairment may be partly independent of nasal congestion, thus explaining oxymetazoline failure in improving olfaction (10).

Therefore, it has been suggested that HCoV, thanks to their neuroinvasive, neurotropic, and neurovirulent properties may be able to induce neuronal impairment (11).

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Infectious Diseases–Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *31 March 2020*
Accepted: *24 April 2020*
Published: *08 May 2020*

Citation:
Bonzano C, Borroni D, Lancia A and Bonzano E (2020) Doxycycline: From Ocular Rosacea to COVID-19 Anosmia. New Insight Into the Coronavirus Outbreak. Front. Med. 7:200. doi: 10.3389/fmed.2020.00200

"Speed and Simplicity" of SARS-CoV-2

Entering into the respiratory tract, all HCoV invade and infect intra-luminal macrophages and epithelial cells. HCoV belong to Coronaviridae, enveloped non-segmented, single-stranded, positive-sense RNA viruses (+)ssRNA. Viral spike (S) proteins manage their cell entry program. They act by binding cell-surface receptors and facilitating the fusion of the virus-cell membrane (12). The spike protein is the key of coronaviruses tropism (12). These S proteins are organized in trimers that end up on the virion in a "corona" way giving it the characteristic crown-like look which seems to play a significative role in viral infection and pathogenesis (12). Similarly to SARS-coronavirus, SARS-CoV-2 seems able to enter in the respiratory epithelium (RE) by binding the human ACE2 receptor. Recombinant S protein has been shown to interact with recombinant ACE2 protein (13). S proteins are the major antigenic determinant managing host immune networks, inhibiting antibodies and the immunity response against the virus by inactivating IFN-α and IFN-ß (12, 14). It is well-established the pivotal role of IFN to protect most tissues from viral pathogenicity. The speed and simplicity of SARS-CoV-2 are typical. Host survival in the presence of the viral infection depends on the efficacy of its IFN system; as a matter of fact, virus survival is linked to its capacity to replicate and spread in the host, by carrying out mechanisms of evasion or subversion of the host IFN response (15).

New Insights Into the Doxycycline Activity

IFNα/β signaling plays a protective role in reducing the virus spread and modulating T cell non-cytolytic antiviral response in limiting viral load. Moreover, some RNA-viruses have developed mechanisms to counteract innate host defense to establish productive infections in their hosts. This is the case of an RNA virus, the vesicular stomatitis virus (VSV) (16).

Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene-5 (Mda-5), seem to have an important role in the recognition of RNA viruses. In particular, it has been shown that immune signaling by RIG-I is involved in the generation of IFN-α/β following VSV infection. Under Dox treatment, cells released high levels of RIG-I proteins eliciting autonomous IFN response, thereby inhibiting viral infection *in vitro* (17).

In another RNA virus, the Respiratory Syncytial Virus (SRV), viral proteins inhibit IFN-α and IFN-β to establish infection (18), and it has been reported a higher expression of interferon-induced protein only after minocycline administration. This suggests an increasing innate immune response supported by tetracycline and the following RSV inhibition (19).

The second-generation tetracycline Dox has an anti-inflammatory and broad spectrum antimicrobial activity (20, 21).

In 1967, Dox was first approved by the FDA (20). It has minimal side effects and it is routinely prescribed for acne and rosacea. Dox is characterized by a ∼100% oral absorption and a prolonged serum half-life (18–22 h) (22).

In ophthalmology, Dox is usually administered in patients affected by ocular rosacea and posterior blepharitis (23). The Dox recommended dose is 40 mg modified release once daily, which could be replaced by minocycline 100 mg, based on patient tolerance or particular requirements (24).

The rationale in its administration is proteolysis inhibition promoted by matrix metalloproteinases (MMPs) (23, 25). MMPs are involved in the regulation of chemical and biological process likes vascular remodeling and angiogenesis (26), so Dox also has anti-angiogenic properties. (27) It regulates cytokines and diminishes neutrophil chemotaxis too (28).

Besides its well-known use in treating bacterial infections, some studies in the literature report that Dox possesses a broad activity against viral infection too (29–31).

The first who described the Dox antiviral effect was Sturtz in 1998 (29), and this suggestion has been confirmed in several followed-up studies. (16, 32, 33)

Topno et al. demonstrated that Dox could interfere with the virion's replication, affecting its structure and causing inhibition of Japanese encephalitis virus-induced pathogenesis *in vitro* (32). The same observation is also reported in a study regarding VSV infection (16) and against the chikungunya virus (CHIKV) (33), suggesting that Dox might interfere with viral replication by aiming proteins essential for these viruses for a successful infection. As proof of that, computational literature reports the Dox ability to bind CHIKV cysteine protease (33), and to exert a significant inhibitory effect on DNV NS2B-NS3 serine protease *in vitro* (30); both these proteases proved to be able to catalyze viral polyproteins cleavage during infection. Moreover, some studies with (+)ssRNA, Dengue virus (DNV), have demonstrated that Dox inhibits virus plaque assembly by interfering with the viral envelope conformational changes needed for virus entry (30). In both CHIKV and DNV, Dox seems to have the ability to bind virus envelop inhibiting viral entry into the cultured cells (30, 33).

Dox proved to be able to markedly decreased the virus-induced cytopathic effect (CPE) and significantly affect viral replication in a dose-dependent manner when used against Porcine Reproductive And Respiratory Syndrome virus (PRRSV) infection in cultured cells (31). Virus mRNA levels were strikingly reduced also in VSV-infected cells in response to Dox; both virus titers and the CPE of VSV infection were significantly influenced by Dox administration in a dose dependent manner (16).

DISCUSSION

Being the olfactory neural system able to regenerate throughout life, it can explain why the recovery of olfaction is common (34).

From our observation, anosmia affected mostly young adults rather than elderly patients, confirming existing findings in the literature (35, 36). It shows up more or less 6 days after fever, cough and muscle aches, but it can be the first and only symptom in many patients, with no mucosal swelling of the olfactory cleft, and that's why we hypothesize that it could be a possible PNS symptom as suggested (2). Among patients affected by PNS symptoms linked to COVID-19, the most common referred were hyposmia, hypogeusia, followed by neuralgia (2). Respiratory viruses such as rhinovirus and parainfluenza Epstein–Barr virus commonly could cause olfactory dysfunction (OD) by leading an inflammation in the olfactory mucosa resulting in rhinorrhea.

FOI 26-3132 - Document 5

Instead, COVID-19 seems to cause an atypical OD as it develops without rhinorrhea or nasal congestion (36).

In 2007, Suzuki et al. identified that coronavirus could be associated with anosmia, and he already speculated that nasal inflammation and related obstruction were not the only etiological factors underlying the OD in viral infection (37). As well-reported in the literature, HCoV could infect peripheral nerve terminals, using the trans-synaptic transfer to access the CNS (36, 38, 39)

In our preliminary observation, the administration of Dox 200 mg once daily seems to improve respiratory symptoms and anosmia under Dox treatment in six patients completely recover after only 2 days of treatment. From our experience, it seems reasonable to continue the treatment at least 8 days. The mean patients' age was 35.8 ± 6.8 years, and 4 (66.7%) were females. One patient reported anosmia as the only COVID-19 manifestation; instead of the other five patients who complained about the loss of smell, in which it appeared 5–7 days after mild fever, dry cough, and malaise. The average time of the recovery COVID-19-linked anosmia after the administration of Dox in these patients was 2.5 ± 0.5 days. We noticed a sudden improvement in all symptoms after the administration of Dox, but our most exciting insight is about the rapid recovery of the smell.

Unlike olfactory sensory neurons (OSNs), nasal epithelium, which includes the respiratory and olfactory epithelium (OE) expresses high levels of ACE2 (40). SARS-CoV-2 seems to target non-neural cell types in the peripheral olfactory system rather than directly enter OSNs, and it seems to be enough to generate cascading damage that could lead to the impairment of OSNs function altering the odor transduction which takes place on their cilia (40). The short-term COVID-19-linked anosmia reported in our experience supports the hypothesis that SARS-CoV2 affects the OE, which can quickly renew and recover following viral clearance (41). The average time to restore the sense of smell, most commonly reported in the literature, lasts from 1–8 days (36), if SARS-COV-2 could directly damage OSNs, recovery should take longer (42). Besides ACE2, Brann et al. also revealed that a cell-surface receptor, CD147, could play a role mediating SARS-CoV-2 cell entry (40). The expression of CD147 is detected in ciliated and goblet cells in the human nasal mucosa (43). Previous reports have shown that Dox has a significant inhibitory effect on CD147 expression (44, 45). Further studies are needed at present to define better if Dox has the ability to inhibiting viral entry by reduced CD147 expression levels. Moreover, thanks to its immunomodulatory and anti-inflammatory properties, Dox could limit the pro-inflammatory state induced by the glial cells activated by the neurotropic virus, ensuring proper epithelial reconstitution in the OE (46, 47). Given the possibility that COVID-19 occurs with the loss of smell and the evidence that corticosteroid may worsen the infection (48), Prof. Claire Hopkins, the British Rhinological Society president, recently suggested avoiding the use of these drugs in the therapeutic approach to the new-onset anosmia during the COVID-19 pandemic, especially if unrelated to previous head trauma or nasal pathology (48).

We are perfectly aware that there is a need for stronger evidence, but our article would intend to underline the importance of considering smell loss as a common symptom of COVID-19, supporting the rationale to treat such patients with Dox based on its interesting antiviral properties.

AUTHOR CONTRIBUTIONS

CB and DB: contributed equally to this manuscript, wrote the article, and reviewed the final version. AL and EB: review and editing of the final manuscript. All authors reviewed the manuscript and agreed with its content.

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FOI 26-3132 - Document 5

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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COMBINATION THERAPY FOR COVID-19 BASED ON IVERMECTIN IN AN AUSTRALIAN POPULATION

TrialSiteNews – October 19, 2021

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Background

The immediacy of "real-world" data in the pre-hospital treatment of Covid-19 using re-purposed drugs has been the keystone to the development of effective therapy in a pandemic situation. The sense of a "common-message database" for the therapeutic use of ivermectin (IVM) collated by clinicians over a time frame of little more than 12 months, involving patients and research workers across geographic and social lines, is extraordinary. From over 20 countries there are 63 controlled studies accepted for meta-analysis, that have included 47,500 subjects with 625 authors.

While this compelling database on the effectiveness of IVM would not be possible without "real-life" studies, at an individual level, these trials give additional value such as information on dosage and combination therapies. They also give confidence to local doctors and regulators as they seek optimal management strategies.

Here, we report successful pre-hospital treatment of Covid-19 patients in Sydney and Melbourne, Australia, using an ivermectin-doxycycline-zinc combination with an important set of observations on symptom resolution and oximetry.

The Study

Six hundred Australian residents with positive PCR symptomatic Covid-19 were treated with a combination of ivermectin (24 mg per day), doxycycline (100 mg bd), and zinc (50mg per day), for 10 days within 48 hrs of obtaining a positive PCR test for Covid-19. The treatment period was from June to September 2021. Seven percent (7%) were given additional vitamins and nutritional supplements. Only 7% identified adverse events from the therapy, mainly minor gut symptoms of nausea, diarrhoea and heartburn. No patient stopped therapy due to adverse drug events.

A subgroup in hotel and home quarantine was available for a more detailed assessment of symptoms and oxygenation status. This cohort of 126 was assessed for 10 symptoms according to a visual analogue scale (ranging from "0" for no symptoms, to "10" for most severe symptoms). Descriptive statistics are summarised in Table 1. P-Values were calculated using Wilcoxon matched-pairs signed-rank t-tests. Chi Squared analysis was used to analyse rates of hospitalization in the 600-patient cohort.

The Outcome

A total of 600 patients with positive PCR tests were treated with the ten-day course of "Ivermectin Triple Therapy" (ITT), which was fully completed in over 90% of those treated. None discontinued therapy as a result of drug side effects. Two visited the hospital for less than 24 hours following a transient arrhythmia and then went home, while five were admitted to hospital (0.83%). There were no deaths. In an equivalent control group of 600, not treated with triple therapy, 70 were admitted to hospital (11.5%), with 6 deaths (1%). Chi-squared analysis of hospitalisation rate shows a significant decrease ($P<0.001$) in the presence of

ivermectin triple therapy intervention. The control data was from contemporary infected subjects in Australia obtained from published Covid Tracking Data.
The subgroup of 126 in quarantine had more detailed documentation. They had an average age of 42 (range: 17-94). The results of symptom analysis are in Table 1. For the 10 symptoms analysed, 98% had a total symptom score severity reduction by the end of the treatment period (P< 0.0001). Symptoms that were most persistent were loss of taste and smell, cough, and fatigue. Oximetry readings in 71 subjects were consistent with mild disease in most (Table 1), though the range extended to the low 70's indicating severe disease in some individuals. At the completion of the 10 -day treatment programme, all subjects had normal oximetry readings.

Comment

This successful study of 600 consecutive subjects treated within 2 days of testing positive on PCR for Covid-19 infection, emphasises the value of early treatment. The results are consistent with the study by Hazan et al, further supporting the value of ITT therapy (ivermectin, doxycycline and zinc).[1] The current study differs from that of Hazan et al who successfully used lower doses of IVM and a shorter treatment duration. The current study also confirms the value of oximetry in monitoring response to therapy, with all hypoxic patients having normal oximetry levels following the treatment protocol. Few become hypoxic in the first 48 hours of symptoms. The rapid increase in oximetry values following IVM, noted by Hazan[1], confirms significant hypoxia was likely avoided in this treated group. Combining oximetry with visual analogue scales to monitor symptoms, is suggested as a valuable tool for future studies.

Visual analogue scale monitoring of symptoms of Covid-19 infection showed a fall from a median total symptom severity score of 37 (range: 0-100) at the onset of treatment to 3 (range: 0-62) at the conclusion of treatment (P<0.0001). The main persistent symptoms were loss of smell/taste, cough and fatigue, recognised to persist for longer periods, however, all displayed significant severity score reductions during the treatment period (P<0.0001). The impact of early treatment with ITT on subsequent "long Covid" will be an important question to pursue. Symptom resolution with untreated Covid-19 is generally 2-4 weeks, emphasising the value of visual analogue scale assessment.

The major differences in admission to hospital and death following ITT therapy compared to contemporary controls is consistent with the large and increasing body of data measuring the impact of ivermectin-combination therapy.[1–3] The results from this study invites comparison with recently announced data by Merck from a study of their re-cycled antiviral polymerase inhibitor, Molnupiravir. The Merck study with 385 treated patients involved a similar group to that treated in the current Australian study judged by near-identical hospital admission and mortality rates in both control groups. In the Merck study, the hospital admission rate was halved (7.3% of treated patients) with no deaths, similar to data for the "first cousin" of Molnupiravir, Favipiravir, which is used extensively in Russia. The important point is that the admission rate in the Merck study is 8-fold greater than the 90% reduction in hospital admissions recorded in the current ITT study (P<0.0001). The public health impact of these findings with a cheap, safe and available therapy, in terms of reduction of load on a health system that will be further stressed as country borders are re-opened, cannot be ignored.
The current study included "real-life" data on consecutive patients thus avoiding selection bias. Therapy was given in two states of Australia, involving over 30 front-line doctors. This compelling data reinforces the value of information from a range of sources and trial methodologies in assessing treatment options, especially in a pandemic situation. The number of patients in this report was capped at 600, as the Australian regulatory body (the TGA)

intervened in the middle of Australia's third Covid wave, to prohibit further prescription of ivermectin for Covid-19 by General Practitioners.

Table 1: Pre and Post Treatment Symptom Severity and Pulse Oximetry Data from Quarantined Individuals (N=126)

	Pre-Treatment			Post-Treatment			
Fever	114	4.2 (4.1)	3 (0-10)	114	0.1 (0.7)	0 (0-5)	<0.0001
Headache	116	5.8 (3.3)	6 (0-10)	116	0.5 (1.1)	0 (0-5)	<0.0001
Cough	120	5.2 (3.3)	5 (0-10)	120	1.1 (1.9)	0 (0-9)	<0.0001
Loss of Taste	111	4.1 (4.5)	2 (0-10)	111	1.8 (3.2)	0 (0-10)	<0.0001
Loss of Smell	112	4.4 (4.5)	2.5 (0-10)	112	2.0 (3.5)	0 (0-10)	<0.0001
Diarrhoea	115	2.3 (3.3)	0 (0-10)	115	0.2 (0.7)	0 (0-5)	<0.0001
Vomiting	114	1.0 (2.7)	0 (0-10)	114	0.03 (0.3)	0 (0-3)	<0.0001
Fatigue	118	6.6 (3.4)	8 (0-10)	118	1.4 (2.0)	0 (0-10)	<0.0001
Muscle Pains	118	5.4 (3.7)	5 (0-10)	118	0.6 (1.4)	0 (0-7)	<0.0001
Joint Pains	114	4.7 (3.9)	5 (0-10)	114	0.4 (1.2)	0 (0-7)	<0.0001
Total Symptom Severity	124	40.9 (26.1)	37 (0-100)	124	7.5 (10.3)	3 (0-62)	<0.0001
Oximetry Reading (%)	71	93.3 (5.0)	95 (71-99)	71	98.1 (1.1)	98 (95-100)	<0.0001

Symptoms were scored on a 0-10 scale with 0 being the absence of associated symptom and 10 being the most severe presentation as reported by the patient. Total Symptom Severity Score was a combined sum of all symptoms assessed and was scored out of 100. Oximetry Readings were scored out of 100%. N = the number of respondents and SD = Standard Deviation. *Non-parametric paired t-tests were performed.

Conclusion

In 'Real Life' this 10-day Ivermectin Triple Therapy reported here, appears to be a very safe, effective and inexpensive early antiviral treatment for Covid-19. Further detail from the more than 30 doctors involved in treating this patient group, will be used for a formal peer-reviewed publication.

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JCI The Journal of Clinical Investigation

P-glycoprotein ABCB1: a major player in drug handling by mammals

Piet Borst, Alfred H. Schinkel

J Clin Invest. 2013;123(10):4131-4133. https://doi.org/10.1172/JCI70430.

Hindsight

Mammalian P-glycoproteins are active drug efflux transporters located in the plasma membrane. In the early nineties, we generated knockouts of the three P-glycoprotein genes of mice, the *Mdr1a*, *Mdr1b*, and *Mdr2* P-glycoproteins, now known as *Abcb1a*, *Abcb1b*, and *Abcb4*, respectively. In the *JCI* papers that are the subject of this Hindsight, we showed that loss of *Mdr1a* (*Abcb1a*) had a profound effect on the tissue distribution and especially the brain accumulation of a range of drugs frequently used in humans, including dexamethasone, digoxin, cyclosporin A, ondansetron, domperidone, and loperamide. All drugs were shown to be excellent substrates of the murine ABCB1A P-glycoprotein and its human counterpart, the MDR1 P-glycoprotein, ABCB1. We found that the ability of ABCB1 to prevent accumulation of some drugs in the brain is a prerequisite for their clinical use, as absence of the transporter led to severe toxicity or undesired CNS pharmacodynamic effects. Subsequent work has fully confirmed the profound effect of the drug-transporting ABCB1 P-glycoprotein on the pharmacokinetics of drugs in humans. In fact, every new drug is now screened for transport by ABCB1, as this limits oral availability and penetration into sanctuaries protected by ABCB1, such as the brain.

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P-glycoprotein ABCB1: a major player in drug handling by mammals

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Mammalian P-glycoproteins are active drug efflux transporters located in the plasma membrane. In the early nineties, we generated knockouts of the three P-glycoprotein genes of mice, the *Mdr1a*, *Mdr1b*, and *Mdr2* P-glycoproteins, now known as *Abcb1a*, *Abcb1b*, and *Abcb4*, respectively. In the *JCI* papers that are the subject of this Hindsight, we showed that loss of *Mdr1a* (*Abcb1a*) had a profound effect on the tissue distribution and especially the brain accumulation of a range of drugs frequently used in humans, including dexamethasone, digoxin, cyclosporin A, ondansetron, domperidone, and loperamide. All drugs were shown to be excellent substrates of the murine ABCB1A P-glycoprotein and its human counterpart, the MDR1 P-glycoprotein, ABCB1. We found that the ability of ABCB1 to prevent accumulation of some drugs in the brain is a prerequisite for their clinical use, as absence of the transporter led to severe toxicity or undesired CNS pharmacodynamic effects. Subsequent work has fully confirmed the profound effect of the drug-transporting ABCB1 P-glycoprotein on the pharmacokinetics of drugs in humans. In fact, every new drug is now screened for transport by ABCB1, as this limits oral availability and penetration into sanctuaries protected by ABCB1, such as the brain.

Generating knockout mice

Generating knockout mice in the early 1990s was quite an adventure. The technology had just been developed, and the homologous recombination required to disrupt genes in mouse embryonic stem cells was still extremely inefficient. We were lucky, however, to work in The Netherlands Cancer Institute, where Hein te Riele and Anton Berns discovered in 1992 that gene targeting in embryonic stem cells could be made much more efficient by using isogenic DNA for transfection rather than DNA from a different mouse strain (1). Nevertheless, generating knockout mice remained a risky undertaking.

After protracted lab discussions, Alfred Schinkel, then a senior postdoc in the Borst lab, took the jump. With hindsight, this was the right decision. Although there were indications that drug-transporting P-glycoproteins could be important in drug pharmacokinetics, it was hard to get conclusive data, as the available inhibitors of P-glycoproteins were still toxic and not very specific. The knockout mice would fill that gap. We reduced the risk of getting scooped by generously explaining to our colleagues elsewhere that we had an edge and would get there first with the expert help of Anton Berns and his knockout crew. Apparently, the competition lost heart, and, in the end, we were the only lab to produce P-glycoprotein knockout mice.

A dead mouse with an unguarded blood-brain barrier

Before we were able to properly analyze the *Mdr1a* (*Abcb1a*) knockout mice, Schinkel received a message from our animal facility that his knockout mice were suddenly dying. When the animal caretakers were asked what they had done, their answer was predictably, "Nothing, we did nothing." It soon emerged, however, that the mice had been sprayed with the pesticide ivermectin to counteract a mite infestation. A toxicity analysis demonstrated that the homozygous *Mdr1a*$^{-/-}$ mice were 100-fold more sensitive to ivermectin than their *Mdr1a*$^{+/+}$ littermates (2). Ivermectin is a strong neurotoxin, but normally it is kept out of the brains of mammals by ABCB1. In the *Mdr1a*$^{-/-}$ mice, about 100-fold more ivermectin accumulated in the brain, leading to lethal neurotoxicity.

This and subsequent work (3–7) established that a drug-transporting P-glycoprotein is an integral part of the blood-brain barrier (BBB) and essential in keeping large, hydrophobic drugs out of the brain. This conclusion was not directly accepted by scientists working on the BBB. Soon after the ivermectin mishap, Piet Borst was invited to address the European BBB Society as a late-breaking speaker. Before the talk, an established BBB researcher explained why large, amphipathic drugs were unable to pass the BBB, using purely chemical arguments, hydrogen bonds, etc. Borst then came with his disappointing message: it is not the subtle chemistry, but a pump that drives the drug from the apical membrane of the cerebral endothelium back into the circulation. This message was not well received. One discussant shouted in desperation, "Did you verify that every single capillary in the brain contains P-glycoprotein?" Of course we had not, but Borst explained that mice without MDR1A P-glycoprotein were "stone dead" after ivermectin administration, indicating that P-glycoprotein should be an essential component of the entire BBB.

The ABCB1 P-glycoprotein also limits uptake of some drugs from the gut

The presence of ABCB1 in the apical membrane of the intestine made it likely that this protein would also impede the uptake of the substrate drugs. It took time before this was proven and even longer before it became accepted that P-glycoprotein is a major factor in the oral availability of some substrate drugs. In fact, when we showed that the MDR1A P-glycoprotein impedes the uptake of digoxin from the gut (5), it was hard to get this ground-breaking result published. Acceptance improved when we obtained the same result for the anticancer drug paclitaxel. In that case, uptake of the drug is nearly completely prevented by P-glycoprotein, making it necessary to administer the drug intravenously (6). The demonstration that P-glycoprotein limits the oral availability of some drugs as well as their penetration into the brain has led to

Conflict of interest: The research groups of the authors receive revenue from commercial distribution of some of the mouse strains mentioned in this paper.

Citation for this article: *J Clin Invest.* 2013;123(10):4131–4133. doi:10.1172/JCI70430.

FOI 26-3132 - Document 5

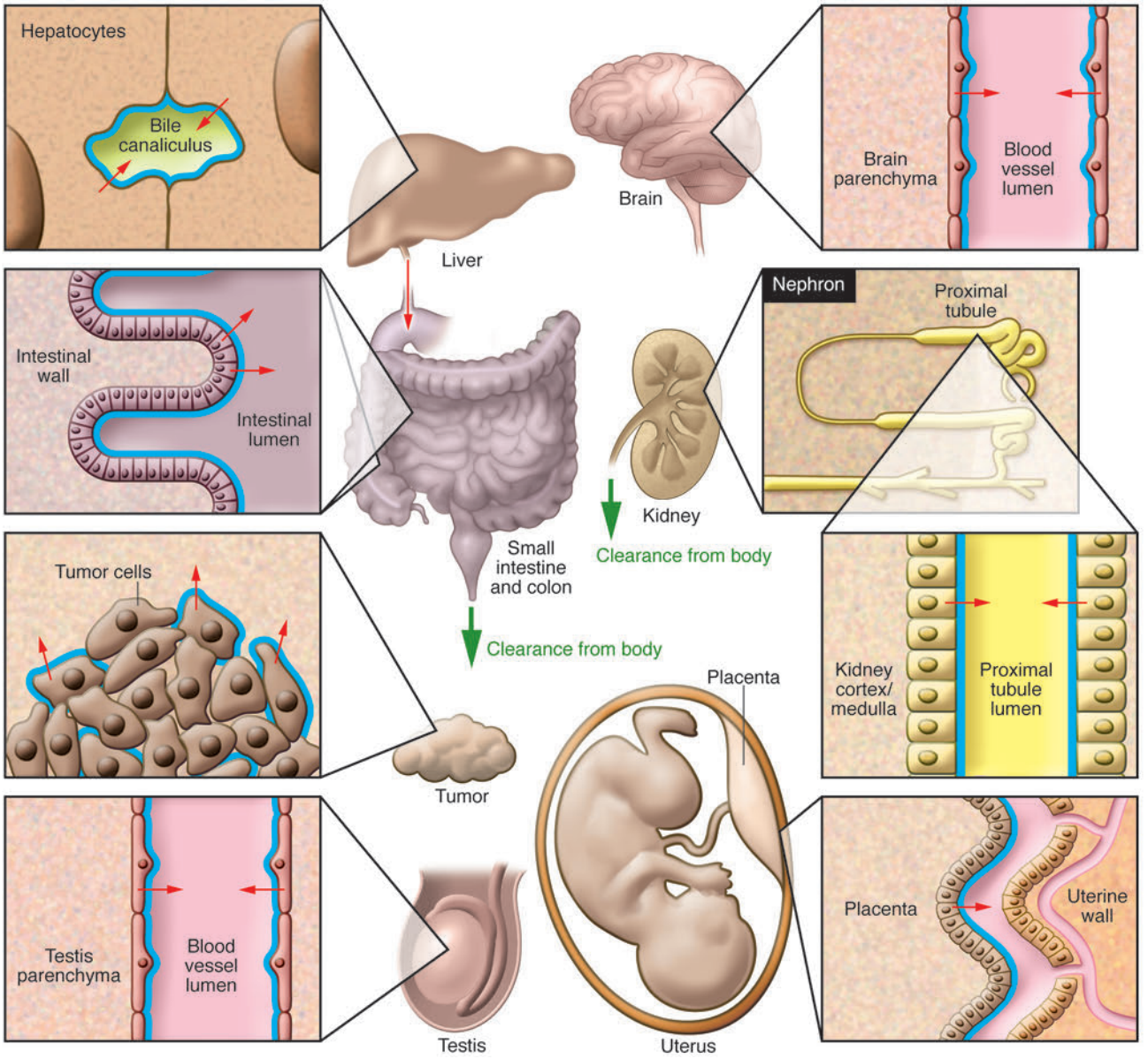


Figure 1
Overview of ABCB1 functional expression throughout the body. Blue lines indicate the location of ABCB1. Small arrows indicate the direction of ABCB1-mediated transport. Bold green arrows indicate net body excretion of ABCB1 substrates. ABCB1 in the BBB and blood-testis and placental fetal-maternal barrier protects brain, testis, and fetus, respectively, from accumulation of ABCB1 substrates. ABCB1 in small and large intestine reduces intestinal uptake (oral availability) and mediates direct intestinal excretion of substrates. ABCB1 in liver and kidney mediates hepatobiliary and renal excretion of substrates. ABCB1 expression in tumor cells can contribute to multidrug resistance.

numerous attempts to eliminate this barrier by using increasingly specific P-glycoprotein inhibitors. P-glycoprotein has also become a buzzword in the pharma industry, as everybody prefers orally available drugs.

Drug-transporting P-glycoprotein today

As the ABCB1 P-glycoprotein is a highly versatile, if not promiscuous, drug transporter, the number of drugs affected by P-glycoprotein has exploded. The number of tissues specifically protected by P-glycoprotein has also increased, as illustrated in Figure 1. Whereas the role of P-glycoprotein in pharmacokinetics is large and undisputed, its role in drug resistance of human tumors has turned out to be much smaller than initially anticipated. There is no doubt that modest amounts of P-glycoprotein can completely protect mouse tumors from substrate drugs, such as taxanes or anthracyclines, but human tumors seem to use this effective defense mechanism only rarely (8).

The knockout approach has been fruitful in pharmacology

After our initial success, we made knockouts of many other transporter protein genes and combined some of these in double knockouts and triple knockouts. Schinkel concentrated on P-glycoprotein and *Abcg2* and, more recently, on drug importers and P450

FOI 26-3132 - Document 5

drug-metabolizing enzymes. Borst knocked out MRPs (ABCCs) (9). Disrupting these genes has generated fundamental insight into the roles of these proteins in physiology and pharmacology. Obviously, eliminating the function of a gene in the germline can lead to secondary adjustments. Indeed, in 1994, we observed some upregulation of the second drug-transporting mouse P-glycoprotein gene, *Abcb1b* (*Mdr1b*), in the *Mdr1a* knockout (2). Thus far, however, these compensatory alterations have not affected the results of our knockout mice in a major way. We have never had reason to regret our entry into the transporter knockout field in 1990, and there is more to come.

Acknowledgments

We are grateful to the Dutch Cancer Society for their continued financial support of most of the work reviewed here. We also thank all our collaborators for their contributions and, especially, Els Wagenaar for her unrelenting dedication and success in generating the early knockout strains.

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FOI 26-3132 - Document 5

American Journal of Therapeutics 28, e434–e460 (2021)

OPEN

Ivermectin for Prevention and Treatment of COVID-19 Infection: A Systematic Review, Meta-analysis, and Trial Sequential Analysis to Inform Clinical Guidelines

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Background: Repurposed medicines may have a role against the SARS-CoV-2 virus. The antiparasitic ivermectin, with antiviral and anti-inflammatory properties, has now been tested in numerous clinical trials.

Areas of uncertainty: We assessed the efficacy of ivermectin treatment in reducing mortality, in secondary outcomes, and in chemoprophylaxis, among people with, or at high risk of, COVID-19 infection.

Data sources: We searched bibliographic databases up to April 25, 2021. Two review authors sifted for studies, extracted data, and assessed risk of bias. Meta-analyses were conducted and certainty of the evidence was assessed using the GRADE approach and additionally in trial sequential analyses for mortality. Twenty-four randomized controlled trials involving 3406 participants met review inclusion.

Therapeutic Advances: Meta-analysis of 15 trials found that ivermectin reduced risk of death compared with no ivermectin (average risk ratio 0.38, 95% confidence interval 0.19–0.73; n = 2438; I^2 = 49%; moderate-certainty evidence). This result was confirmed in a trial sequential analysis using the same DerSimonian–Laird method that underpinned the unadjusted analysis. This was also robust against a trial sequential analysis using the Biggerstaff–Tweedie method. Low-certainty evidence found that ivermectin prophylaxis reduced COVID-19 infection by an average 86% (95% confidence interval 79%–91%). Secondary outcomes provided less certain evidence. Low-certainty evidence suggested that there may be no benefit with ivermectin for "need for mechanical ventilation,"

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The preprint of this review received no funding. This updated version was funded by the crowdfunding initiative https://www.gofundme.com/f/help-us-get-lifesaving-drug-approved-for-covid19

The authors have no conflicts of interest to declare.

T. A. Lawrie and A. Bryant cowrote the review; they also sifted the search and classified studies for inclusion and entered and checked the data in RevMan and performed analyses. Data extraction was divided among T. A. Lawrie, A. Bryant, and T. Dowswell. T. Dowswell and A. Bryant graded the evidence. E. J. Fordham prepared the text on ivermectin mechanisms, use in pregnancy, and among the elderly. S. R. Hill prepared the brief economic commentary. Clinicians S. Mitchell and T. C. Tham contributed to the interpretation of the evidence in the discussion and conclusions. All authors reviewed and approved the final version of the manuscript.

This article discusses off-label use of the FDA-approved medication ivermectin against COVID-19.

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FOI 26-3132 - Document 5

whereas effect estimates for "improvement" and "deterioration" clearly favored ivermectin use. Severe adverse events were rare among treatment trials and evidence of no difference was assessed as low certainty. Evidence on other secondary outcomes was very low certainty.

Conclusions: Moderate-certainty evidence finds that large reductions in COVID-19 deaths are possible using ivermectin. Using ivermectin early in the clinical course may reduce numbers progressing to severe disease. The apparent safety and low cost suggest that ivermectin is likely to have a significant impact on the SARS-CoV-2 pandemic globally.

Keywords: ivermectin, prophylaxis, treatment, COVID-19, SARS-CoV-2

INTRODUCTION

To date, very few treatments have been demonstrated to reduce the burden of morbidity and mortality from COVID-19. Although corticosteroids have been proven to reduce mortality in severe disease,[1] there has been little convincing evidence on interventions that may prevent disease, reduce hospitalizations, and reduce the numbers of people progressing to critical disease and death.

Ivermectin is a well-known medicine that is approved as an antiparasitic by the World Health Organization and the US Food and Drug Administration. It is widely used in low- and middle-income countries (LMICs) to treat worm infections.[2,3] Also used for the treatment of scabies and lice, it is one of the World Health Organization's Essential Medicines.[4] With total doses of ivermectin distributed apparently equaling one-third of the present world population,[5] ivermectin at the usual doses (0.2–0.4 mg/kg) is considered extremely safe for use in humans.[6,7] In addition to its antiparasitic activity, it has been noted to have antiviral and anti-inflammatory properties, leading to an increasing list of therapeutic indications.[8]

Since the start of the SARS-CoV-2 pandemic, both observational and randomized studies have evaluated ivermectin as a treatment for, and as prophylaxis against, COVID-19 infection. A review by the Front Line COVID-19 Critical Care Alliance summarized findings from 27 studies on the effects of ivermectin for the prevention and treatment of COVID-19 infection, concluding that ivermectin "demonstrates a strong signal of therapeutic efficacy" against COVID-19.[9] Another recent review found that ivermectin reduced deaths by 75%.[10] Despite these findings, the National Institutes of Health in the United States recently stated that "there are insufficient data to recommend either for or against the use of ivermectin for the treatment of COVID-19,"[11] and the World Health Organization recommends against its use outside of clinical trials.[12]

Ivermectin has exhibited antiviral activity against a wide range of RNA and some DNA viruses, for example, Zika, dengue, yellow fever, and others.[13] Caly et al[14] demonstrated specific action against SARS-CoV-2 in vitro with a suggested host-directed mechanism of action being the blocking of the nuclear import of viral proteins[14,15] that suppress normal immune responses. However, the necessary cell culture EC_{50} may not be achievable in vivo.[16] Other conjectured mechanisms include inhibition of SARS-CoV-2 3CLPro activity[17,18] (a protease essential for viral replication), a variety of anti-inflammatory effects,[19] and competitive *binding* of ivermectin with the viral S protein as shown in multiple *in silico* studies.[20] The latter would inhibit viral binding to ACE-2 receptors suppressing infection. Hemagglutination via viral binding to sialic acid receptors on erythrocytes is a recently proposed pathologic mechanism[21] that would be similarly disrupted. Both host-directed and virus-directed mechanisms have thus been proposed, the clinical mechanism may be multimodal, possibly dependent on disease stage, and a comprehensive review of mechanisms of action is warranted.

Developing new medications can take years; therefore, identifying existing drugs that can be repurposed against COVID-19 that already have an established safety profile through decades of use could play a critical role in suppressing or even ending the SARS-CoV-2 pandemic. Using repurposed medications may be especially important because it could take months, possibly years, for much of the world's population to get vaccinated, particularly among LMIC populations.

Currently, ivermectin is commercially available and affordable in many countries globally.[6] A 2018 application for ivermectin use for scabies gives a direct cost of $2.90 for 100 12-mg tablets.[22] A recent estimate from Bangladesh[23] reports a cost of US$0.60—US$1.80 for a 5-day course of ivermectin. For these reasons, the exploration of ivermectin's potential effectiveness against SARS-CoV-2 may be of particular importance

FOI 26-3132 - Document 5

for settings with limited resources.[24] If demonstrated to be effective as a treatment for COVID-19, the cost-effectiveness of ivermectin should be considered against existing treatments and prophylaxes.

The aim of this review was to assess the efficacy of ivermectin treatment among people with COVID-19 infection and as a prophylaxis among people at higher risk of COVID-19 infection. In addition, we aimed to prepare a brief economic commentary (BEC) of ivermectin as treatment and as prophylaxis for COVID-19.[25]

METHODS

The conduct of this review was guided by a protocol that was initially written using Cochrane's rapid review template and subsequently expanded to a full protocol for a comprehensive review.[26]

Search strategy and selection criteria

Two reviewers independently searched the electronic databases of Medline, Embase, CENTRAL, Cochrane COVID-19 Study Register, and Chinese databases for randomized controlled trials (RCTs) up to April 25, 2021 (see **Appendix 1–3**, **Supplemental digital content 1**, http://links.lww.com/AJT/A95); current guidance[25] for the BEC was followed for a supplementary search of economic evaluations. There were no language restrictions, and translations were planned to be performed when necessary.

We searched the reference list of included studies, and of two other 2021 literature reviews on ivermectin,[9] as well as the recent WHO report, which included analyses of ivermectin.[12] We contacted experts in the field (Drs. Andrew Hill, Pierre Kory, and Paul Marik) for information on new and emerging trial data. In addition, all trials registered on clinical trial registries were checked, and trialists of 39 ongoing trials or unclassified studies were contacted to request information on trial status and data where available. Many preprint publications and unpublished articles were identified from the preprint servers MedRχiv and *Research Square*, and the International Clinical Trials Registry Platform. This is a rapidly expanding evidence base, so the number of trials are increasing quickly. Reasons for exclusion were recorded for all studies excluded after full-text review.

Data analysis

We extracted information or data on study design (including methods, location, sites, funding, study author declaration of interests, and inclusion/exclusion criteria), setting, participant characteristics (disease severity, age, gender, comorbidities, smoking, and occupational risk), and intervention and comparator characteristics (dose and frequency of ivermectin/comparator). The primary outcome for the intervention component of the review included death from any cause and presence of COVID-19 infection (as defined by investigators) for ivermectin prophylaxis. Secondary outcomes included time to polymerase chain reaction (PCR) negativity, clinical recovery, length of hospital stay, admission to hospital (for outpatient treatment), admission to ICU or requiring mechanical ventilation, duration of mechanical ventilation, and severe or serious adverse events, as well as post hoc assessments of improvement and deterioration. All of these data were extracted as measured and reported by investigators. Numerical data for outcomes of interest were extracted according to intention to treat.

If there was a conflict between data reported across multiple sources for a single study (eg, between a published article and a trial registry record), we contacted the authors for clarification. Assessments were conducted by 2 reviewers (T.L., T.D., A.B., or G.G.) using the Cochrane RCT risk-of-bias tool.[27] Discrepancies were resolved by discussion.

Continuous outcomes were measured as the mean difference and 95% confidence intervalss (CI), and dichotomous outcomes as risk ratio (RR) and 95% CI.

We did not impute missing data for any of the outcomes. Authors were contacted for missing outcome data and for clarification on study methods, where possible, and for trial status for ongoing trials.

We assessed heterogeneity between studies by visual inspection of forest plots, by estimation of the I^2 statistic ($I^2 \geq 60\%$ was considered substantial heterogeneity),[28] by a formal statistical test to indicate statistically significant heterogeneity,[29] and, where possible, by subgroup analyses (see below). If there was evidence of substantial heterogeneity, the possible reasons for this were investigated and reported. We assessed reporting biases using funnel plots if more than 10 studies contributed to a meta-analysis.

We meta-analyzed data using the random effects model (DerSimonian and Laird method)[30] using RevMan 5.4.1 software.[27,31] The results used the inverse variance method for weighting.[27] Some sensitivity analyses used other methods that are outlined below and some calculations were performed in *R*[32] through an interface[33] to the *netmeta* package.[34] Where possible, we performed subgroup analyses grouping trials by disease severity, inpatients versus outpatients, and single dose versus multiple doses. We performed sensitivity analyses by excluding studies at high risk of bias. We conducted further post hoc sensitivity analyses using alternative methods to test the robustness of results in the presence of zero events in both arms in a number of trials[35] and estimated odds ratios [and additionally RR for the Mantel–Haenszel

FOI 26-3132 - Document 5

(MH) method] using a fixed effects model. The models incorporate evidence from single-zero studies without having to resort to continuity corrections. However, double-zero studies are excluded from the analysis; so, the risk difference was also assessed using the MH method as this approach can adequately incorporate trials with double-zero events. This method can also use a random-effects component. A "treatment-arm" continuity correction was used, where the values 0.01, 0.1, and 0.25 were added where trials reported zero events in both arms. It has been shown that a nonfixed continuity correction is preferable to the usual 0.5.[35] Other methods are available but were not considered due to difficulty in interpretation, sensitivity of assumptions, or the fact they are rarely used in practice.[36–40]

Trial sequential analysis

When a meta-analysis is subjected to repeated statistical evaluation, there is an exaggerated risk that "naive" point estimates and confidence intervals will yield spurious inferences. In a meta-analysis, it is important to minimize the risk of making a false-positive or false-negative conclusion. There is a trade-off between the risk of observing a false-positive result (type I error) and the risk of observing a false-negative result (type II error). Conventional meta-analysis methods (eg, in RevMan) also do not take into account the amount of available evidence. Therefore, we examined the reliability and conclusiveness of the available evidence using trial sequential analyses (TSA).[41–43] The DerSimonian–Laird (DL) method was used because this is most often used in meta-analytic practice and was also used in the primary meta-analysis.[30]

The TSA was used to calculate the required information size (IS) to demonstrate or reject a relative reduction in the risk (RRR) of death in the ivermectin group, as found in the primary meta-analysis. We assumed the estimated event proportion in the control group from the meta-analysis because this is the best and most representative available estimate. Recommended type I and II error rates of 5% and 10% were used, respectively (power of 90%),[43] powering the result on the effect observed in the primary meta-analyses. We did not identify any large COVID-19 trials powered on all-cause mortality, so powering on some external meaningful difference was not possible. Any small RRR is meaningful in this context, given the scale of the pandemic, but the required IS would be unfeasibly high for this analysis if powered on a small difference. The only reliable data on ivermectin in its repurposed role for treatment against COVID-19 will be from the primary meta-analysis. Therefore, assuming it does not widely deviate from other published systematic reviews, a pragmatic decision was therefore made to power on the pooled meta-analysis effect estimate for all-cause mortality a priori. This is more reflective of a true meaningful difference. We used a model variance-based estimate to correct for heterogeneity. A continuity correction of 0.01 was used in trials that reported zero events in one or both arms. The required IS is the sample size required for a reliable and conclusive meta-analysis and is at least as large as that needed in a single powered RCT. The heterogeneity corrected required IS was used to construct sequential monitoring boundaries based on the O'Brien–Fleming type alpha-spending function for the cumulative z-scores (corresponding to the cumulative meta-analysis),[43] analogous to interim monitoring in an RCT, to determine when sufficient evidence had been accrued. These monitoring boundaries are relatively insensitive to the number of repeated significance tests. They can be used to further contextualize the original meta-analysis and enhance our certainty around its conclusions. We used a two-sided test, so also considered futility boundaries (to test for no statistically significant difference) and the possibility that ivermectin could harm. Sensitivity analyses were performed excluding the trial of Fonseca,[44] which was a cause of substantial heterogeneity (but retained in the core analysis because it was at low risk of bias). Its removal dramatically reduced I^2 and D^2 (diversity) estimates, thus reducing the model variance-based estimate to correct for heterogeneity. Two further sensitivity analyses were performed using 2 alternative random effect models, namely the Biggerstaff–Tweedie (BT) and Sidik–Jonkman (SJ) methods.[43]

All outcomes have been assessed independently by 2 review authors (T.D. and A.B.) using the GRADE approach,[45] which ranks the quality and certainty of the evidence. The results of the TSAs will also form part of the judgment for the primary all-cause mortality outcome. The results are presented in a summary of findings table. Any differences in judgments were resolved by discussion with the wider group. We used Cochrane Effective Practice and Organisation of Care guidance to interpret the evidence.[46]

RESULTS

Search results and risk-of-bias assessment

The combined and preliminary deduplicated total was n = 583. We also identified 11 records from other sources (reference lists, etc). See PRISMA flow diagram for inclusion and exclusion details of these references (Figure 1).

FOI 26-3132 - Document 5

The supplementary search for the BEC identified 17 studies, of which 4 were retrieved in full. No full trial- or model-based economic evaluations (cost–utility analyses, cost–effectiveness analyses, or cost–benefit analyses) were identified.

Twenty-one trials in treatment and 2 trials in prophylaxis of COVID-19 met review inclusion. One further study[47] reported separate treatment and prophylaxis components; we label this study "Elgazzar" under both questions. In effect, there were 22 trials in treatment and 3 in prophylaxis. All of these contributed data to at least one review outcome and meta-analysis. Fifteen trials contributed data for the primary outcome for ivermectin treatment (death); 3 studies reported the primary outcome for prophylaxis (COVID-19 infection). Characteristics of included studies are given in Table 1. Seventeen studies[47–63] were excluded as they were not RCTs and we identified 39 ongoing studies[64–102] and 2 studies[103,104] are awaiting classification.

A risk-of-bias summary graph is given in Figure 2. Eleven studies[23,24,44,47,105,106–111] used satisfactory random sequence generation and allocation concealment. Two trials described satisfactory sequence generation, but it was unclear whether allocation was concealed.[112,113]

Ten trials reported adequate blinding of the participants/personnel and/or the outcome assessors.[23,24,44,105,107,109,110,111,113,114] The others were either unclear or high risk for blinding. We considered blinding to be a less important criterion for evaluation of evidence related to the review's primary outcomes, namely death and laboratory-confirmed COVID-19 infection, which are objective outcomes.

We did not consider publication on preprint web sites to constitute a risk of bias because all studies were scrutinized and peer reviewed by us during the review process and, where additional information was needed, we contacted the authors for clarification.

Main findings

Twenty-four RCTs (including 3 quasi-RCTs) involving 3406 participants were included, with sample sizes ranging from 24 to 476 participants. Twenty-two trials in treatment and 3 trials in prophylaxis met review inclusion, including the trial of Elgazzar et al, which reported both components. For trials of COVID-19 treatment, 16 evaluated ivermectin among participants with mild to moderate COVID-19 only; 6 trials included patients with severe COVID-19. Most compared ivermectin with placebo or no ivermectin; 3 trials included an active comparator (Table 1). Three RCTs involving 738 participants

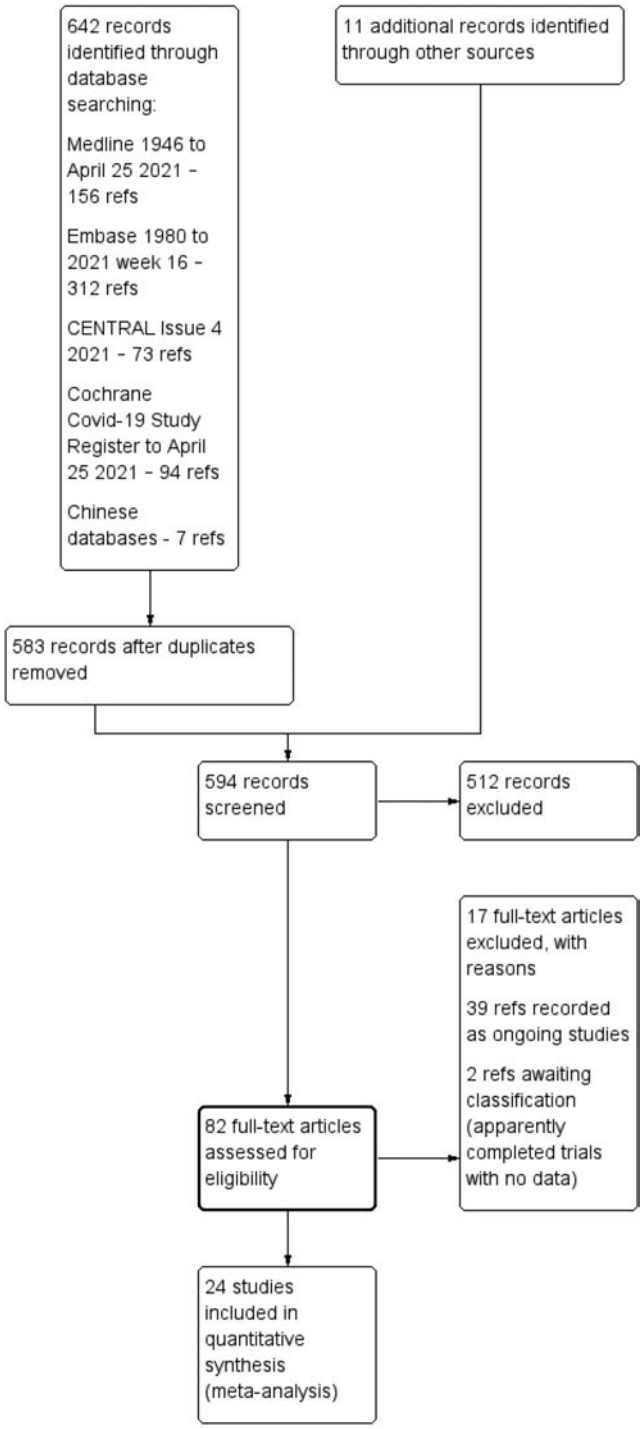


FIGURE 1. Study flow diagram from search on 25 April 2021.

were included in the prophylaxis trials. Most trials were registered, self-funded, and undertaken by clinicians working in the field. There were no obvious conflicts of interest noted, with the exception of two trials.[85,139]

FOI 26-3132 - Document 5

Table 1. Summary of study characteristics.

Study ID	Country	Design	Funding	Participants	Sample size	Ivermectin dose and frequency*	Comparator	Origin of data	Main outcomes reported
COVID-19 treatment studies									
Ahmed 2020[23]	Bangladesh	Double-blind	BPL(Pharma); Bangladesh, Canada, Sweden, and UK govt	Mild to moderate COVID (inpatients)	72	12 mg × 1 day or × 5 days (3 study arms)*	Placebo	Published in PR journal; emailed/ responded with data	Time to viral clearance (PCR –ve), remission of fever and cough within 7 days, duration of hospitalization, mortality, failing to maintain sats >93%, adverse events, PCR –ve at 7 and 14 days
Babalola 2020[105]	Nigeria	Double-blind	Self-funded	Asymptomatic, mild or moderate COVID (45 inpatients and 17 outpatients)	62	6 mg every 84 hrs × 2 wks (arm 1) or 12 mg every 84 hrs × 2 wks (arm 2)	Ritonavir/lopinavir	MedRxiv preprint: emailed/ responded with data. Paper accepted for publication	Time to PCR –ve, laboratory parameters (platelets, lymphocytes, clotting time), clinical symptom parameters
Bukhari 2021[135]	Pakistan	Open-label	None reported	Mild to moderate COVID (inpatients)	100	12 mg × 1 dose	SOC	MedRxiv preprint	Viral clearance, any adverse side effects, mechanical ventilation
Chaccour 2020[24]	Spain	Double-blind	Idapharma, ISGlobal, and the University of Navarra	Mild COVID (outpatients)	24	0.4 mg/kg × 1 dose	Placebo	Published in PR journal	PCR +ve at day 7, proportion symptomatic at day 4,7,14,21, progression, death, adverse events
Chachar 2020[112]	Pakistan	Open-label	Self-funded	Mild COVID (outpatients)	50	12 mg at 0, 12, and 24 hours (3 doses)	SOC	Published in PR journal	Symptomatic at day 7
Chowdhury 2020[136]	Bangladesh	Quasi-RCT	None reported	Outpatients with a +ve PCR (approx. 78% symptomatic)	116	0.2 mg/kg x1 dose*	HCQ 400 mg 1st day then 200 mg BID × 9 days + AZM 500 mg daily × 5 days	Research square preprint	Time to –ve PCR test; period to symptomatic recovery; adverse events
Elgazzar 2020[47]	Egypt	RCT	None reported	Mild to severe COVID (inpatients)	200	0.4 mg/kg daily × 4 days	HCQ 400 mg BID × 1 day then 200 mg BID × 9 days	Research square preprint: emailed/ responded with data	Improved, progressed, died. Also measured CRP, D-dimers, HB, lymphocyte, serum ferritin after one week of treatment
Fonseca 2021[44]	Brazil	Double-blind	Institution-funded	Moderate to severe (inpatients)	167	14 mg daily × 3 days (plus placebos × 2 additional days)	HCQ—400 mg BID on day 0 then daily × 4 days; CQ -450 mg BID day 0 then daily × 4 days	Prepublication data/ manuscript in progress obtained via email	Death, invasive mechanical ventilation
Gonzalez 2021[137]	Mexico	Double-blind	Institution-funded	Moderate to severe (inpatients)	108	12 mg × 1 dose	Placebo	MedRxiv preprint	Length of hospital stay, invasive mechanical ventilation, death, time to negative PCR
Hashim 2020[138]	Iran	Quasi-RCT	None reported	Mild to critical (inpatients)	140	0.2 mg/kg × 2 days* Some had a 3rd dose a week later	SOC	MedRxiv preprint	Death, mean time to recovery, disease progression (deterioration)
Krolewiecki 2020[106]	Argentina	Open-label	None reported	Mild to moderate (inpatients)	45	0.6 mg/kg/d × 5 days	Placebo	Research Gate and SSRN preprints	Viral load reduction in respiratory secretions day 5, IVM concentrations in plasma, severe adverse events
Lopez-Medina 2021[85]	Columbia	Double-blind	Institution-funded	Mild (outpatients)	476	0.3 mg/kg elixir × 5 days	Placebo	Published in a PR journal	Resolution of symptoms within 21 days, deterioration, clinical condition, hospitalization, adverse events
Mahmud 2020[107]	Bangladesh	Double-blind	None reported	Mild to moderate COVID (inpatients)	363	12 mg × 1 dose*	Placebo + SOC	Data published on clinical trial registry and clarification obtained via email	Improvement, deterioration, late clinical recovery, persistent PCR test +ve

(*Continued on next page*)

FOI 26-3132 - Document 5

Table 1. (*Continued*) Summary of study characteristics.

Study ID	Country	Design	Funding	Participants	Sample size	Ivermectin dose and frequency*	Comparator	Origin of data	Main outcomes reported
Mohan 2021[110]	India	Double-blind	Institution-funded	Mild to moderate	152	12 mg or 24 mg elixir × 1 dose	Placebo	MedRxiv preprint research	Conversion of RT-PCR to negative result, decline of viral load at day 5 from enrollment
Niaee 2020[108]	Iran	Double-blind	Institution-funded	Mild to severe COVID	180	0.2 mg/kg × 1 and 3 other dosing options) ~ 14 mg tablet†	Placebo	Research Square preprint	Deaths, length of stay, biochemical parameters
Okumus 2021[115]	Turkey	Quasi-RCT	None reported	Severe COVID	66	0.2 mg/kg × 5 days	SOC	Prepublication data/ manuscript in progress obtained via email	Clinical improvement, deterioration, death, SOFA scores
Petkov 2021[139]	Bulgaria	Double-blind	Pharma-funded	Mild to moderate COVID	100	0.4 mg/kg × 3 days	Placebo	Prepublication data obtained from another source	Rate of conversion to PCR negative
Podder 2020[140]	Bangladesh	Open-label	Self-funded	Mild to moderate (outpatients)	62	0.2 mg/kg × 1 dose	SOC	Published in PR journal	Duration of symptoms, recovery time to symptom free from enrollment, recovery time to symptom free from symptom onset, repeat PCR result on day 10
Raad 2021[113]	Lebanon	Double-blind	Self-funded	Asymptomatic outpatients	100	9 mg PO if 45 kg–64 kg, 12 mg PO if 65 kg–84 kg and 0.15 mg/kg if body weight ≥85 kg	Placebo	Prepublication data/ manuscript in progress obtained via email	Viral load reduction, hospitalization, adverse effects
Ravikirti 2021[109]	India	Double-blind	Self-funded	Mild to moderate COVID (inpatients)	112	12 mg × 2 days + SOC	Placebo + SOC	Published in PR journal	A negative RT-PCR report on day 6, symptomatic on day 6, discharge by day 10, admission to ICU, need for invasive mechanical ventilation, mortality
Rezai 2020[111]	Iran	Double-blind	None reported	Mild to moderate (inpatient)	60	0.2 mg/kg × 1 dose	SOC	Prepublication data obtained from another source	Clinical symptoms, respiratory rate and O2 saturation
Schwartz 2021[114,141]	Israel	Double-blind	None reported	Mild to moderate (outpatients)	94	0.15–0.3 mg/kg × 3 days	Placebo	Prepublication data obtained from another source	Viral clearance at day 4, 6, 8 and 10), hospitalization
COVID-19 prophylaxis studies									
Chahla 2021[142]	Argentina	Open-label	None reported	Health care workers	234	12 mg (in drops) weekly + iota-carrageenan 6 sprays daily × 4 wk	SOC	Prepublication data/ manuscript in progress obtained via email	COVID-19 infection (not clear if measured by PCR or symptoms)
Elgazzar 2020[47]	Egypt	Open-label	Self-funded	Health care and family contacts	200	0.4 mg/kg, weekly × 2 weeks	SOC	Research square preprint: emailed/ responded with data	Positive PCR test
Shouman 2020[143]	Egypt	Open-label	Self-funded	Family contacts	304	2 doses (15–24 mg depending on weight) on day 1 and day 3	SOC	Published in PR journal	Symptoms and/or positive COVID-19 PCR test within 14 days; adverse events

*Also administered doxycycline.
†multiarm trial.
SOC, standard of care; PR, peer review.

FOI 26-3132 - Document 5

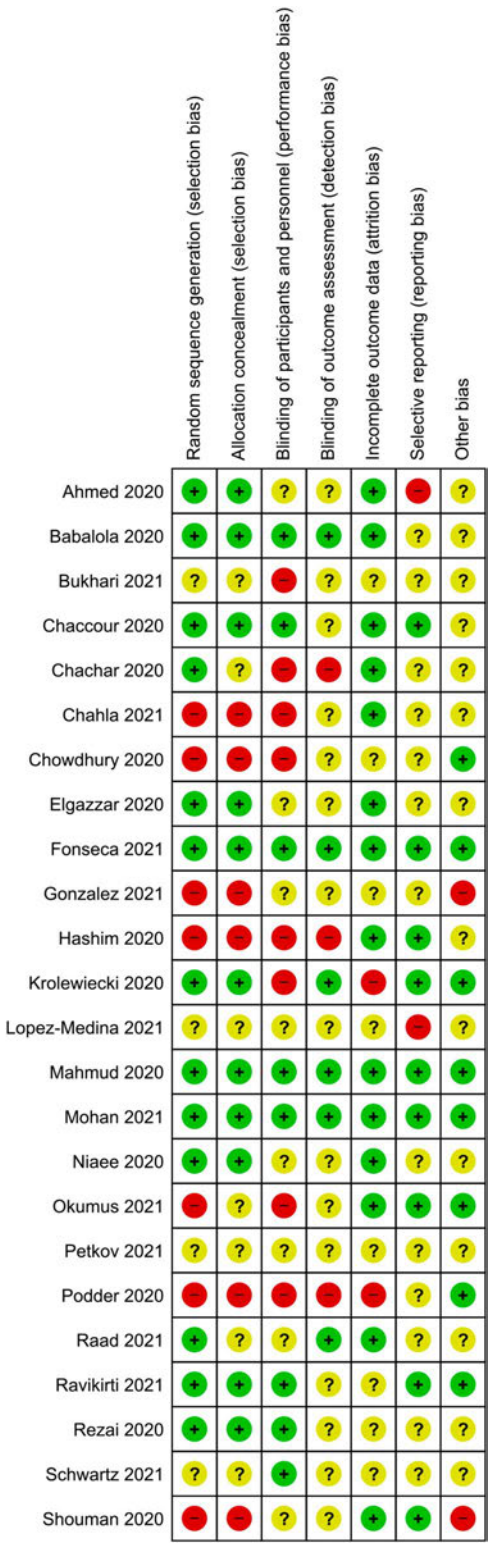


FIGURE 2. Risk-of-bias summary: review authors' judgments about each risk of bias item for each included study.

Ivermectin treatment versus no ivermectin treatment

Twenty-two trials (2668 participants) contributed data to the comparison ivermectin treatment versus no ivermectin treatment for COVID-19 treatment.

All-cause mortality

Meta-analysis of 15 trials, assessing 2438 participants, found that ivermectin reduced the risk of death by an average of 62% (95% CI 27%–81%) compared with no ivermectin treatment [average RR (aRR) 0.38, 95% CI 0.19 to 0.73; $I^2 = 49\%$]; risk of death 2.3% versus 7.8% among hospitalized patients in this analysis, respectively (SoF Table 2 and Figure 3). Much of the heterogeneity was explained by the exclusion of one trial[44] in a sensitivity analysis (average RR 0.31, 95% CI 0.17–0.58, n = 2196, $I^2 = 22\%$), but because this trial was at low risk of bias, it was retained in the main analysis. The source of heterogeneity may be due to the use of active comparators in the trial design. The results were also robust to sensitivity analyses excluding 2 other studies with an active treatment comparator (average RR 0.41, 95% CI 0.23–0.74, n = 1809, $I^2 = 8\%$). The results were also not sensitive to the exclusion of studies that were potentially at higher risk of bias (average RR 0.29, 95% CI 0.10–0.80, 12 studies, n = 2095, $I^2 = 61\%$), but in subgroup analysis, it was unclear as to whether a single dose would be sufficient. The effect on reducing deaths was consistent across mild to moderate and severe disease subgroups. Subgrouping data according to inpatient and outpatient trials was not informative because few outpatient studies reported this serious outcome. The conclusions of the primary outcome were also robust to a series of alternative post hoc analyses that explored the impact of numerous trials that reported no deaths in either arm. Extreme sensitivity analyses using a treatment arm continuity correction of between 0.01 and 0.5 did not change the certainty of the evidence judgments (Table 3).

Trial sequential analysis

TSA, using the DL random-effects method, showed that there may have been sufficient evidence accrued before the end of 2020 to show significant benefit of ivermectin over control for all-cause mortality. The cumulative z-curve in Figure 8 crossed the trial sequential monitoring boundaries after reaching the required IS, implying that there is firm evidence for a beneficial effect of ivermectin use over no ivermectin use in mainly hospitalized participants with mild to moderate COVID-19 infection.

Table 2. Summary of findings table of ivermectin versus no ivermectin for COVID-19 treatment in any setting.

Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	No. of participants (studies)	Quality of the evidence (GRADE)
	Assumed risk No ivermectin	Corresponding risk Ivermectin			
Death from any cause	78 per 1000 (all disease severity)	48 fewer deaths per 1000 (21–63)	RR = 0.38 (0.19–0.73)	2438 (15)	Moderate†
Recovery time to negative PCR test, in days	Absolute risks were not computed due to certainty of evidence being low and, in some cases, number of events being sparse		MD = −3.20 (−5.99 to −0.40)	375 (6)	Very low†,‡,§
Time to clinical recovery, in days (outpatients)			MD = −1.06 (−1.63 to −0.49)	176 (2)	Very low†,‡,§
Time to clinical recovery, in days (mild to moderate COVID-19 inpatients)			MD = −7.32 (−9.25 to −5.39)	96 (1)	Very low†,¶
Time to clinical recovery, in days (severe COVID-19 inpatients)			MD = −3.98 (−10.06 to 2.10)	33 (1)	Very low†,¶
Admission to ICU			RR=1.22 (0.75–2.00)	379 (2)	Very low¶,‖
Need for mechanical ventilation			RR=0.66 (0.14–3.00)	431 (3)	Low§,‖
Length of hospital stay, in days			MD= 0.13 (−2.04 to 2.30)	68 (1)	Very low†,¶
Admission to hospital			RR 0.16 (0.02–1.32)	194 (2)	Very low†,¶
Duration of mechanical ventilation	Not reported				
Improvement (mild to moderate COVID-19)*	635 improved per 1000	159 more per 1000 (from 51 more to 286 more)	RR 1.25 (1.08–1.45)	681 (5)	Low†,‡
Deterioration (any disease severity)	143 per 1000	93 fewer per 1000 (from 50 fewer to 116 fewer)	RR 0.35 (0.19–0.65)	1587 (7)	Low†,‡
Serious adverse events	7/867 (0.8%) had an SAE in ivermectin group and 2/666 (0.3%) in control		RR=1.65 (0.44–6.09)	1533 (11)	Low†,‡

*Only one study contributed to the "severe" COVID-19 subgroup and subgroup data were not pooled due to subgroup differences.
†Downgraded −1 for study design limitations.
‡Downgraded −1 for inconsistency.
§Downgraded −1 for imprecision.
¶Downgraded −2 for imprecision/sparse data.
‖Downgraded −1 for indirectness.

The TSA was used to calculate the IS required to demonstrate or reject a 62% RRR of death in the ivermectin group, as observed in the primary meta-analysis. This estimate is similar to effect estimates reported in other reviews.[10] We assumed a 7.8% event proportion in the control group, which was the average control group

FOI 26-3132 - Document 5

Table 3. Sensitivity analyses for death from any cause considering methods for dealing with zero events in trials.

Method	Measure	Model	Effect size (95% CI)	Details
Peto	OR	FE	0.35 (0.24 to 0.53)	Handles single-zero trials
M-H	OR	FE	0.37 (0.24 to 0.56)	Handles single-zero trials
M-H	OR	RE	0.33 (0.16 to 0.68)	Handles single-zero trials
M-H	RR	FE	0.42 (0.29 to 0.60)	Handles single-zero trials
M-H	RR	RE	0.37 (0.19 to 0.74)	Handles single-zero trials
M-H	RD	FE	−0.04 (−0.06 to −0.02)	Handles double-zero trials
M-H	RD	RE	−0.03 (−0.06 to −0.00)	Handles double-zero trials
IV	RD	FE	−0.01 (−0.02 to −0.00)	Handles double-zero trials
IV	RD	RE	−0.02 (−0.04 to −0.00)	Handles double-zero trials
Treatment arm continuity correction methods using IV			Accounting for double zeros	Accounting for all zeros
0.01	RR	FE	0.54 (0.36 to 0.79)	0.58 (0.39–0.88)
0.01	RR	RE	0.43 (0.25 to 0.72)	0.58 (0.39–0.88)
0.1	RR	FE	0.54 (0.37 to 0.79)	0.56 (0.38–0.84)
0.1	RR	RE	0.43 (0.26 to 0.73)	0.46 (0.26–0.80)
0.25	RR	FE	0.54 (0.37 to 0.79)	0.55 (0.37–0.81)
0.25	RR	RE	0.44 (0.26 to 0.73)	0.45 (0.26–0.76)
0.5	RR	FE	0.54 (0.37 to 0.79)	0.55 (0.35–0.78)
0.5	RR	RE	0.45 (0.27 to 0.74)	0.47 (0.29–0.75)

FE, fixed effects; IV, inverse variance; M-H, Mantel-Haenszel; RD, risk difference; RE, random effects; TACC, treatment arm continuity correction.

event rate from the primary meta-analysis. We used a model variance-based estimate of 49.1% (diversity estimate) to correct for heterogeneity. The required IS was 1810 participants (Figure 8), which was exceeded by the total number of observed participants in the meta-analysis (n = 2438). In the TSA plots, the red dashed lines in Figure 8 represent the trial sequential monitoring boundaries using the O'Brien–Fleming alpha-spending function. The solid blue line is the cumulative z-curve and represents the observed trials in the cumulative meta-analysis. The adjusted significance boundaries for the cumulative z-curve were constructed under the assumption that significance testing may have been performed each time a new trial was added to the meta-analysis. In Figure 8, the z-curve crosses the boundary after reaching the required IS, thereby supporting the previous conclusion in RevMan 5.4.1[31] using the DL method that ivermectin is superior to control in reducing the risk of death.

Sensitivity analyses

Sensitivity analysis excluding the trial of Fonseca[44] significantly reduced heterogeneity in the meta-analysis and thus the diversity estimate in the TSA using the DL model. This strengthened the suggestion in the primary core analysis that the required IS had been reached (Figure 9). Because the DL estimator could potentially underestimate the between-trials variance,[43] we performed further sensitivity analyses using 2 alternative random-effects model approaches. The results of the primary TSA analysis were robust to sensitivity analysis using the BT method with the same parameters, excluding the Fonseca[44] trial, which was a cause of substantial heterogeneity (Figure 10). The TSA

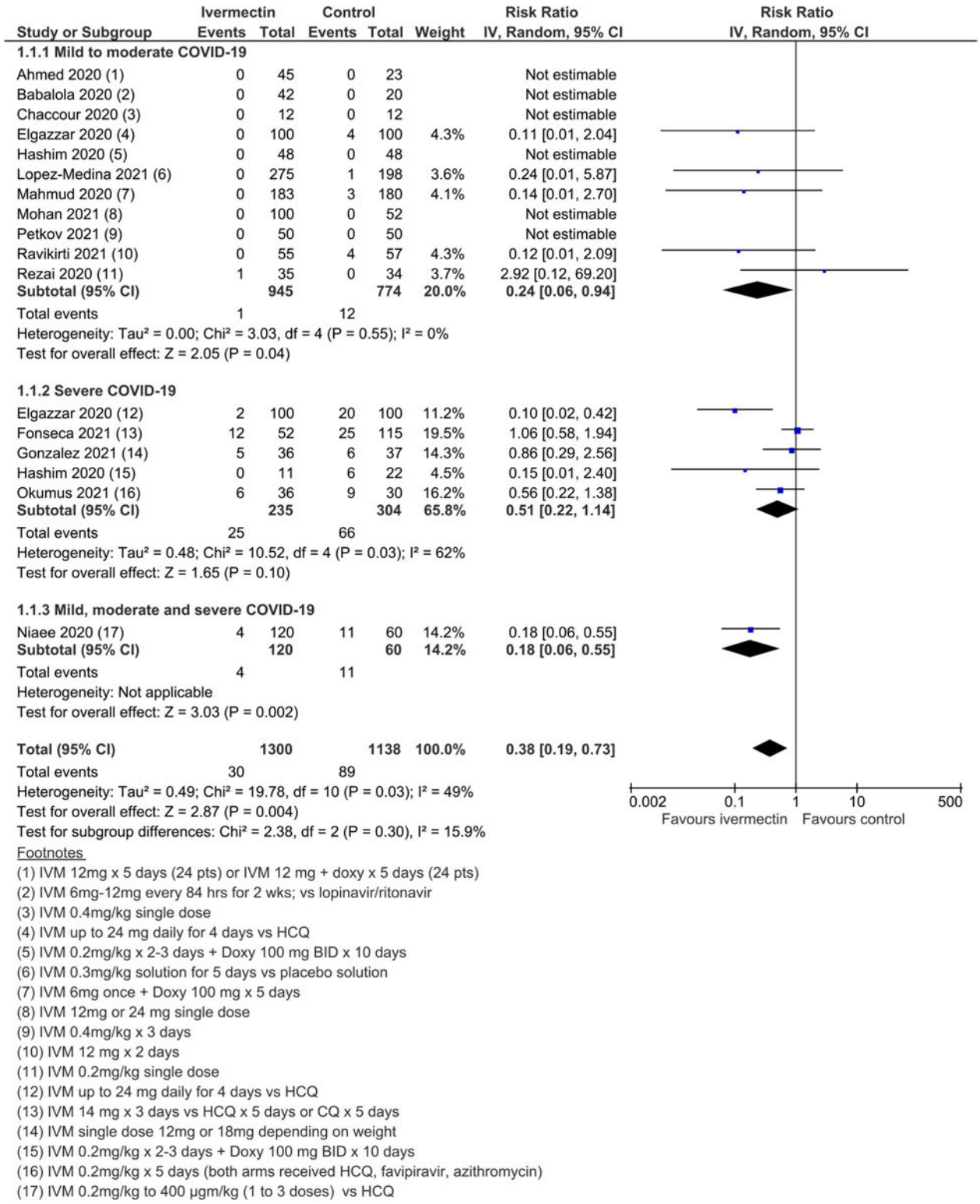


FIGURE 3. Death due to any cause.

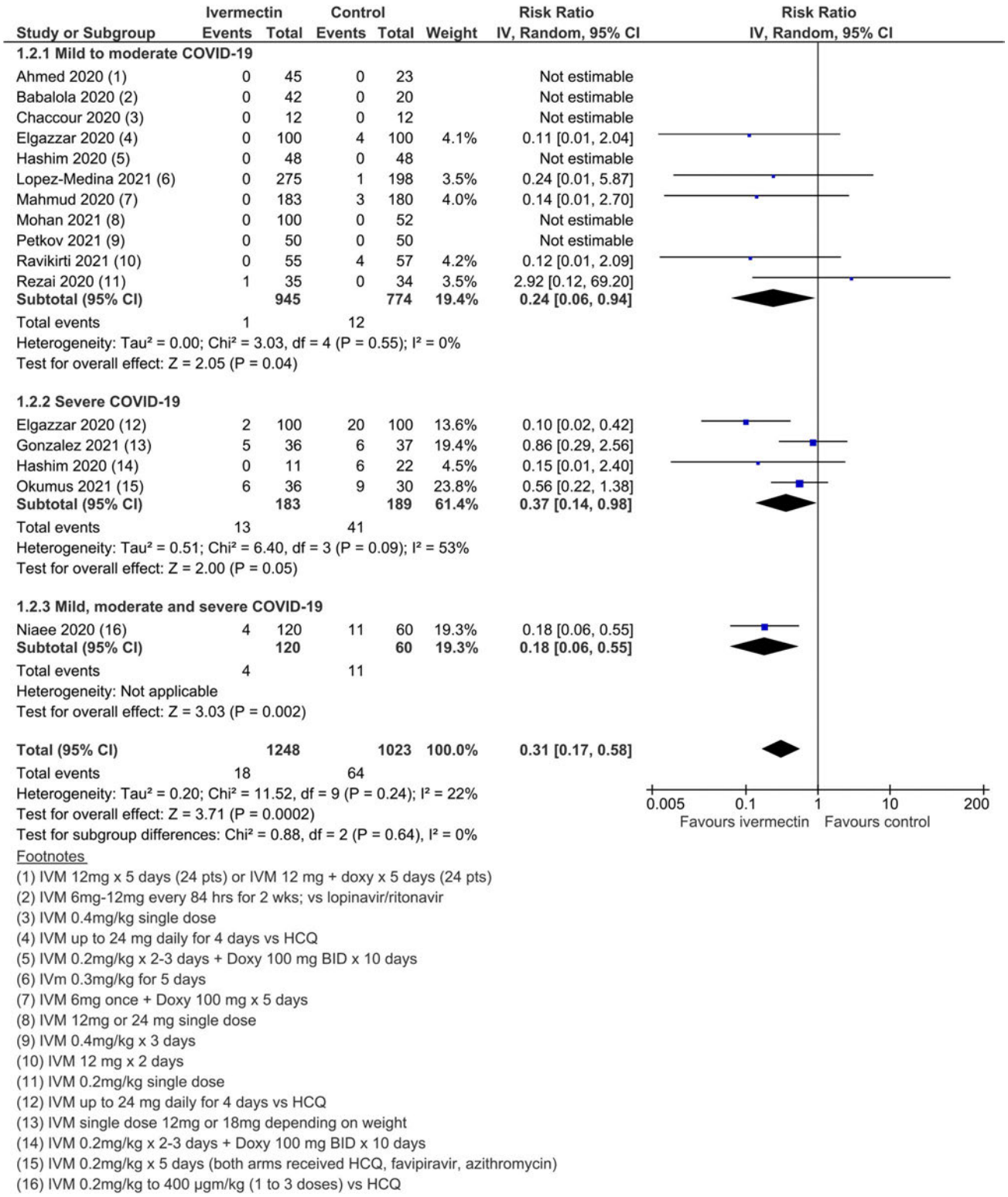


FIGURE 4. Death due to any cause, excluding an outlier study responsible for the heterogeneity.

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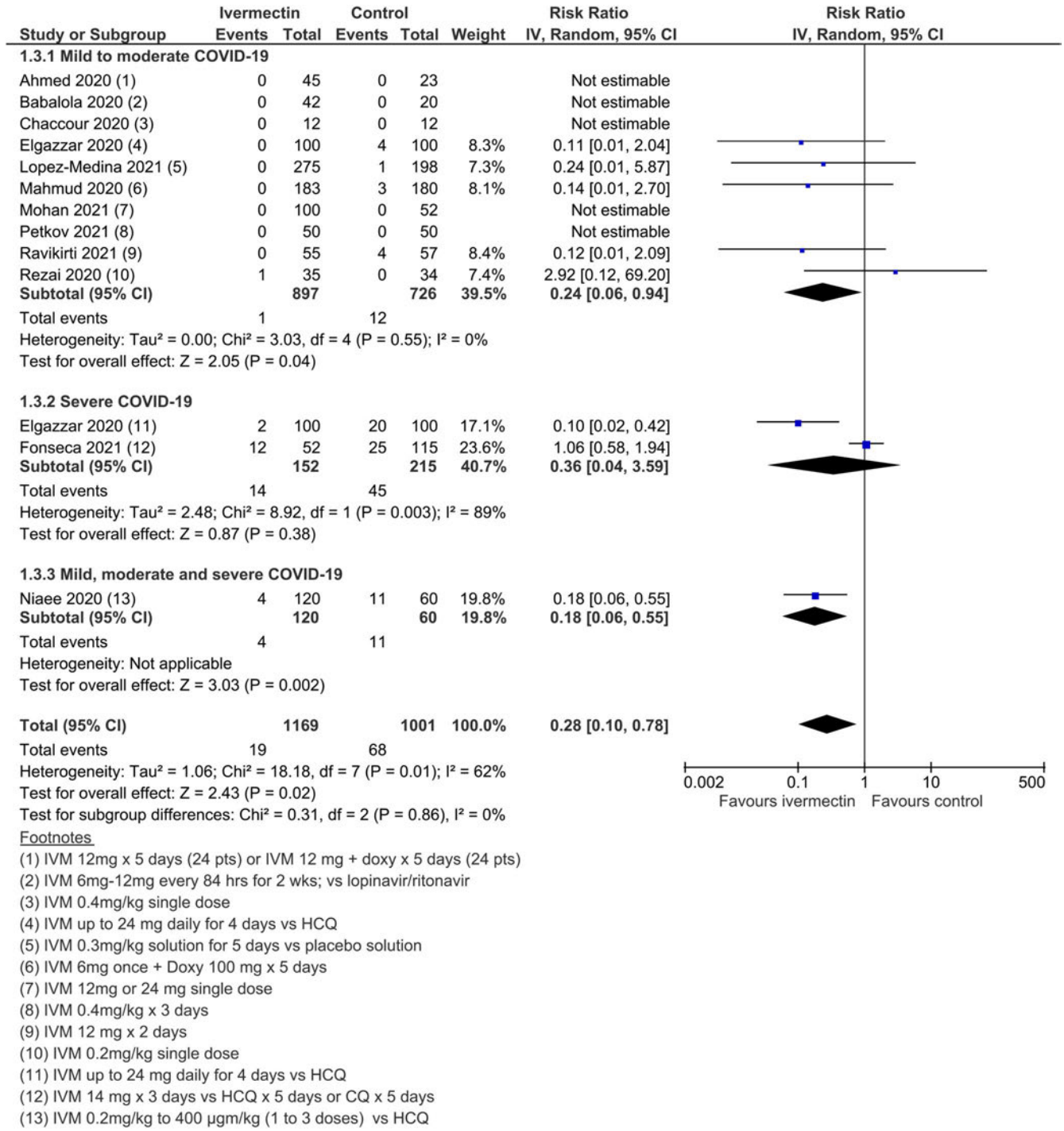


FIGURE 5. Death due to any cause, excluding high risk-of-bias studies.

comprehensively confirms the result of the conventional meta-analysis. The required IS was 1064.

The required IS was not reached in the TSA using the SJ method, largely because diversity from the model was high (Figure 11). The SJ estimator may overestimate the between-trials variance in meta-analyses with mild heterogeneity, thus producing artificially wide confidence intervals.[43] When the diversity estimate was reduced to the same as in the DL model, the required IS was reached in the SJ model (data not shown). There was no evidence of futility using the SJ method in any scenario.

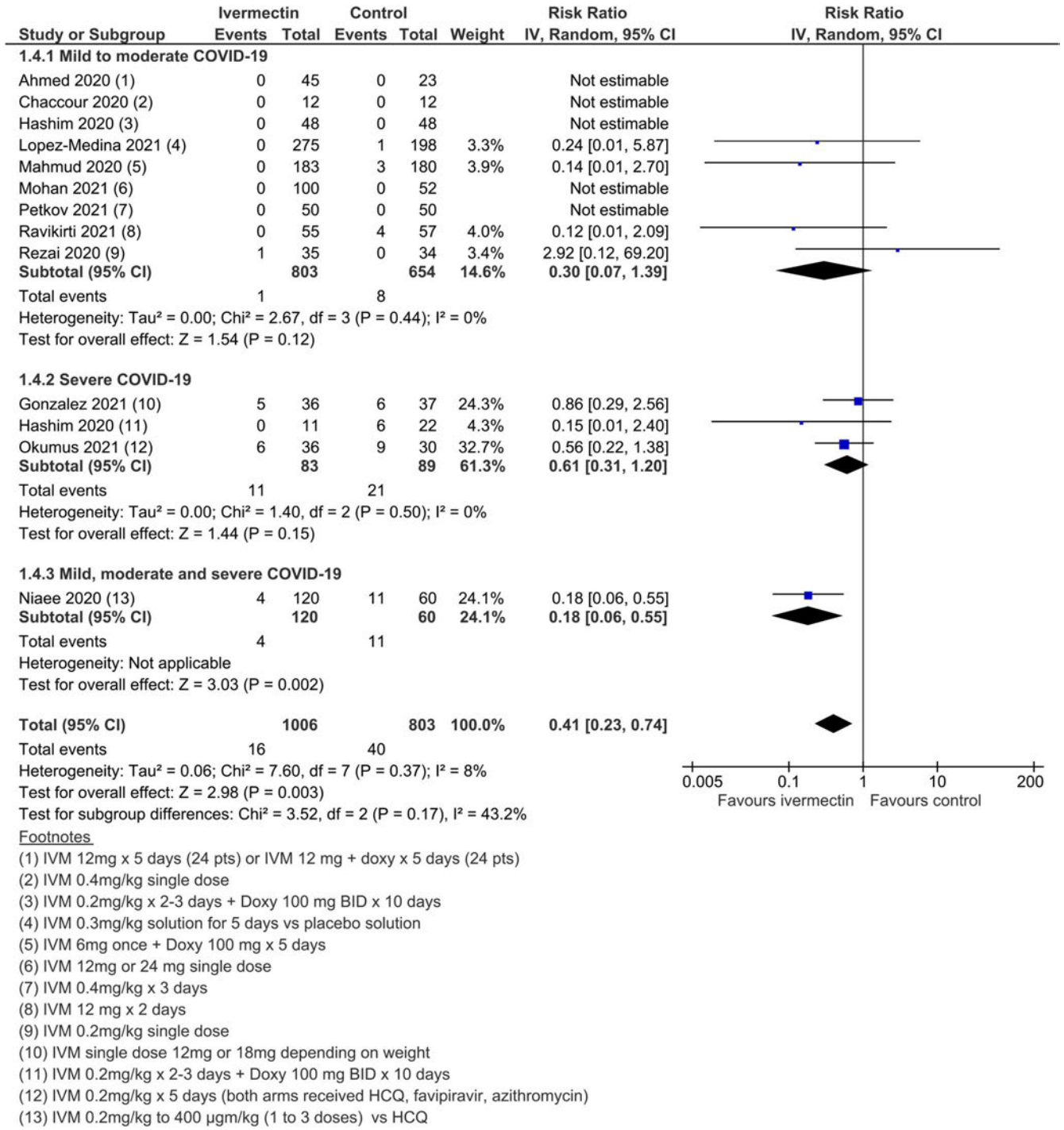


FIGURE 6. Death due to any cause, excluding studies with active controls.

Certainty of the evidence for all-cause mortality

Overall, death from any cause, taking into account all composite analyses, was judged to provide moderate-certainty evidence (SoF Table 2 and Figures 4–11). A funnel plot corresponding to the primary outcome of death from any cause did not seem to suggest any evidence of publication bias (Figure 7). Furthermore, the ease with which trial reports can be uploaded as preprints should reduce this risk.

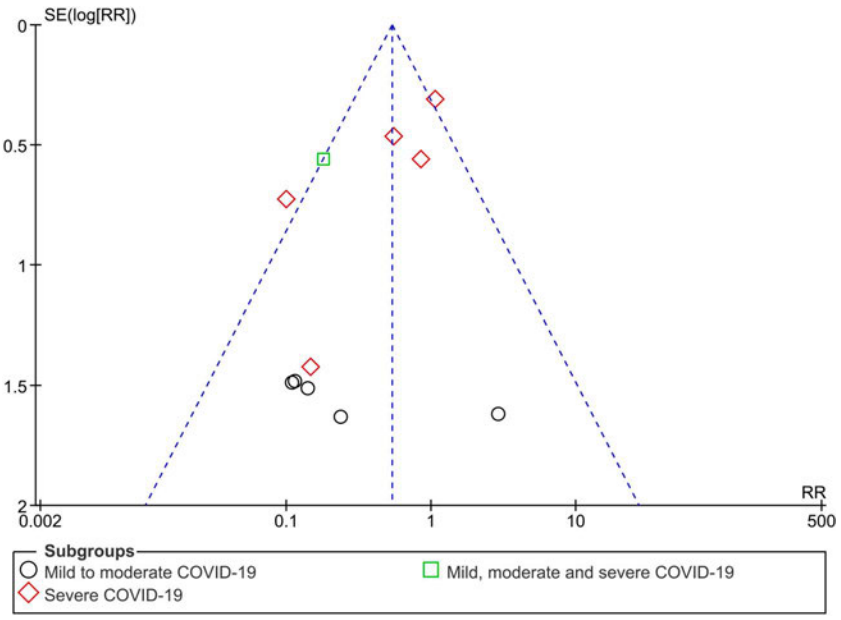


FIGURE 7. Funnel plot of ivermectin versus control for COVID-19 treatment for all-cause death (subgrouped by severity).

Secondary outcomes

Secondary outcomes provided low to very low certainty evidence (SoF Table 2). Low-certainty findings suggested that there may be no benefit with ivermectin for "need for mechanical ventilation," whereas effect estimates for "improvement" and "deterioration" favored ivermectin but were graded as low certainty due to study design limitations and inconsistency (Figures 12–14). All other secondary outcome findings were assessed as very low certainty.

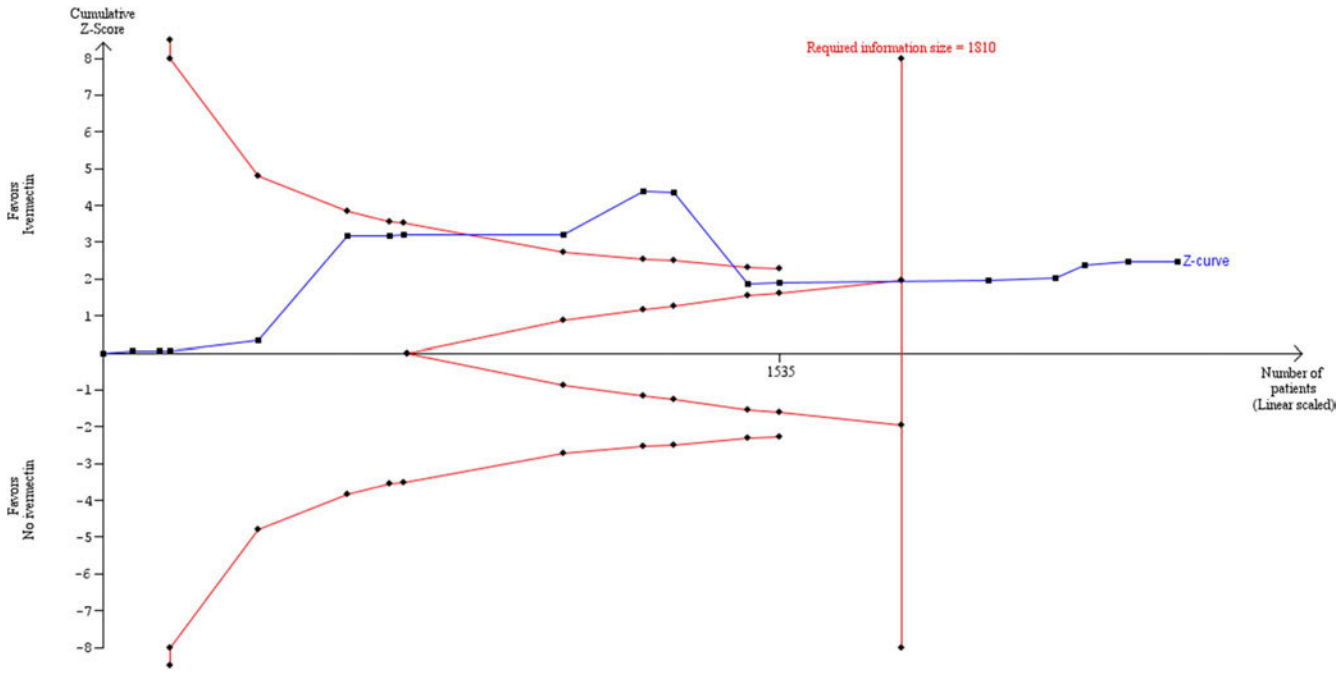


FIGURE 8. Trial sequential analysis using DL random-effects method with parameter estimates of $\alpha = 0.05$, $\beta = 0.1$, control rate = 7.8%, RRR = 62%, and diversity = 49.5%.

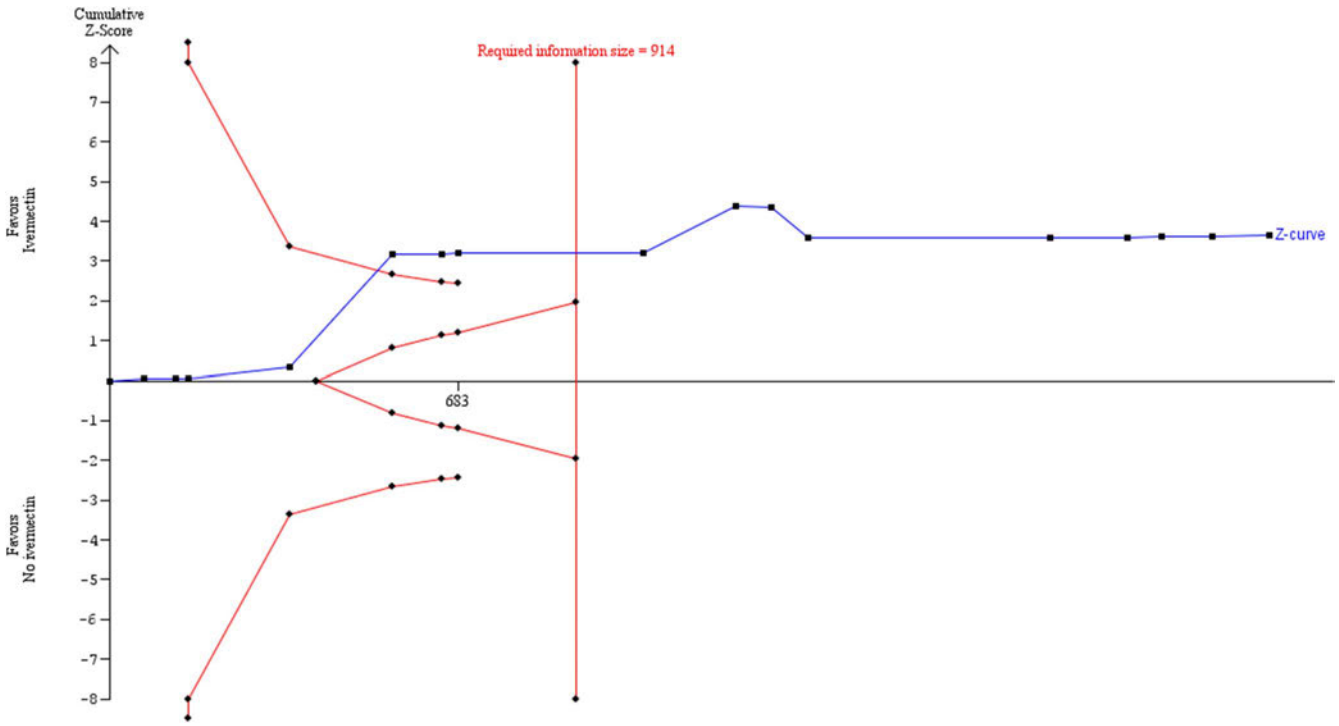


FIGURE 9. Sensitivity analysis excluding an outlier study responsible for the heterogeneity, showing trial sequential analysis using DL random-effects method with parameter estimates of $\alpha = 0.05$, $\beta = 0.1$, control rate = 7.8%, = 62%, and diversity = 0%.

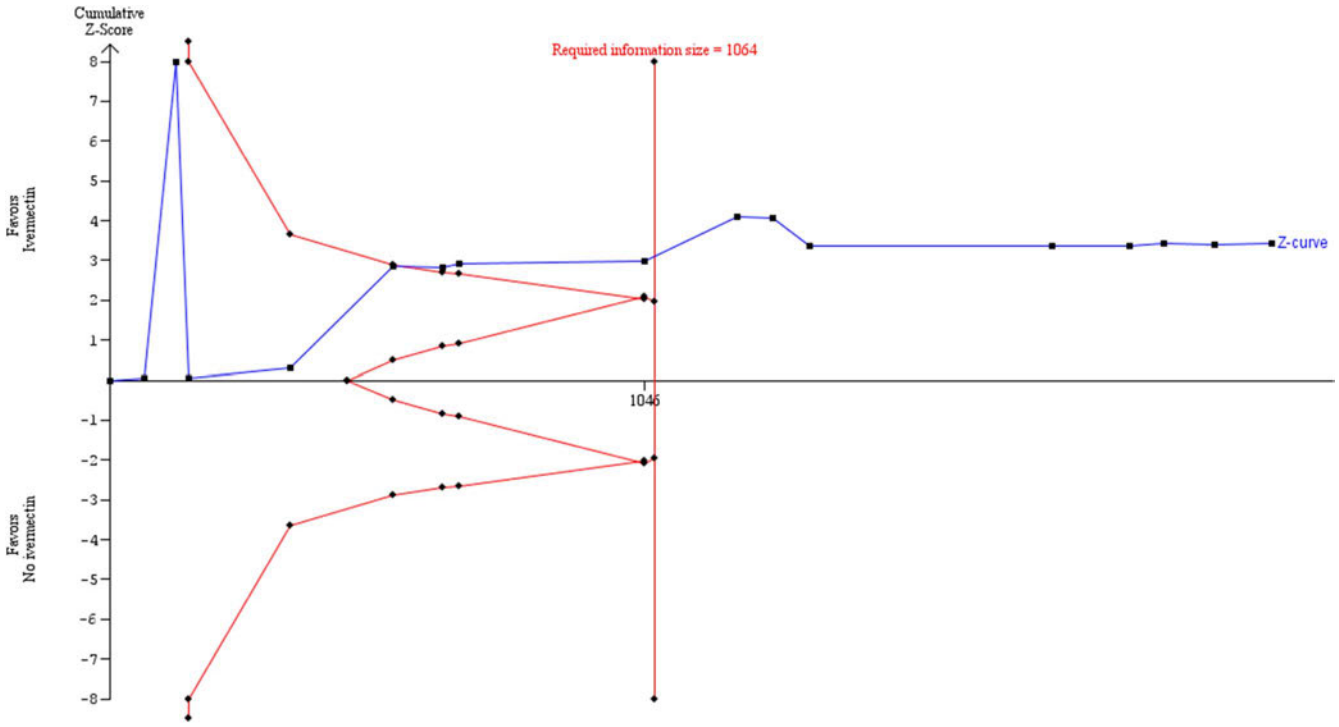


FIGURE 10. Sensitivity analysis excluding an outlier study responsible for the heterogeneity, showing trial sequential analysis using Biggerstaff–Tweedie random-effects method with parameter estimates of $\alpha = 0.05$, $\beta = 0.1$, control rate = 7.8%, RRR = 62%, and diversity = 14.2%.

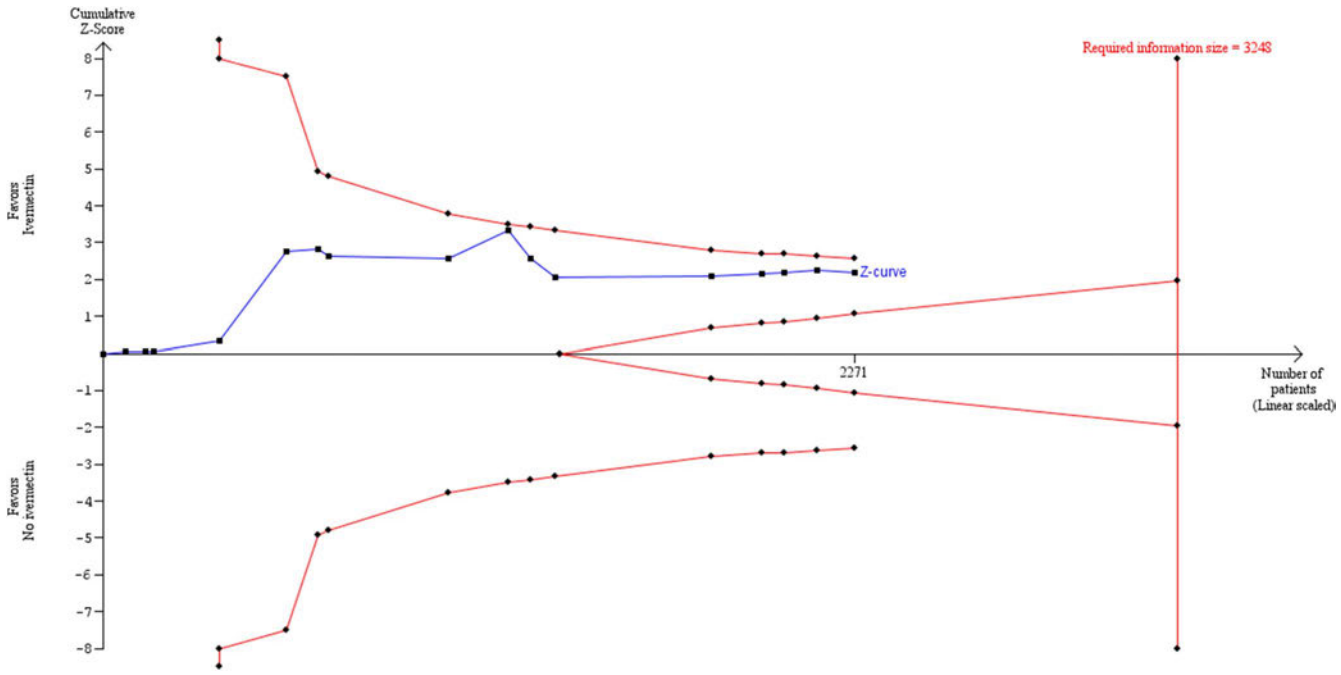


FIGURE 11. Sensitivity analysis excluding an outlier study responsible for the heterogeneity, showing trial sequential analysis using Sidik–Jonkman random-effects method with parameter estimates of $\alpha = 0.05$, $\beta = 0.1$, control rate = 7.8%, RRR = 62%, and diversity = 71.9%.

Meta-analysis of 11 trials, assessing 1533 participants, found that there was no significant difference between ivermectin and control in the risk of severe adverse events (aRR 1.65, 95% CI 0.44–6.09; $I^2 = 0\%$; *low certainty evidence*, downgraded for imprecision and study design limitations). Seven severe adverse events were reported in the ivermectin group and 2 in controls. The SAEs were as follows: 2 patients in the Mahmud trial[107] had esophagitis (this is a known side effect of doxycycline, which was coadministered with ivermectin in this trial); one patient in the study by Krolewiecki et al[106] had hyponatremia (this trial used high-dose ivermectin for 5 days); and 2 patients in a study from Turkey[115] had serious "delirium-like behavior, agitation, aggressive attitude, and altered state of consciousness," which the authors attributed to metabolic insufficiencies in MDR-1/ABCB1 or CYP3A4 genes, screening for which was a study feature. In the Lopez-Medina et al[85] trial, there were 2 SAEs in each arm (SoF Table 2).

Ivermectin prophylaxis versus no ivermectin prophylaxis

Three studies involving 738 participants evaluated ivermectin for COVID-19 prophylaxis among health care workers and COVID-19 contacts. Meta-analysis of these 3 trials, assessing 738 participants, found that ivermectin prophylaxis among health care workers and COVID-19 contacts probably reduces the risk of

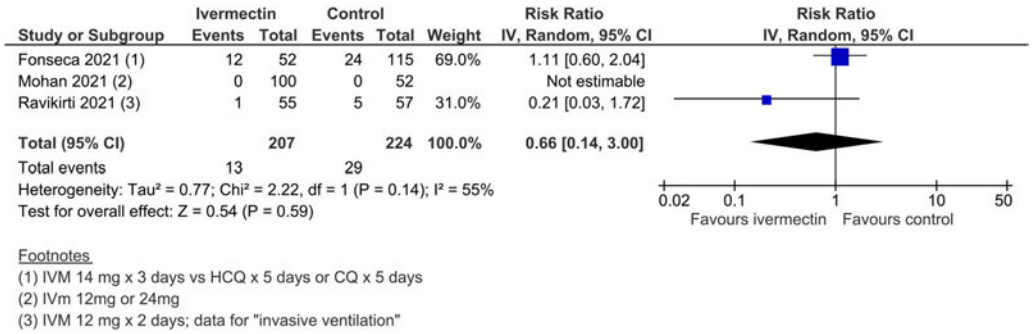
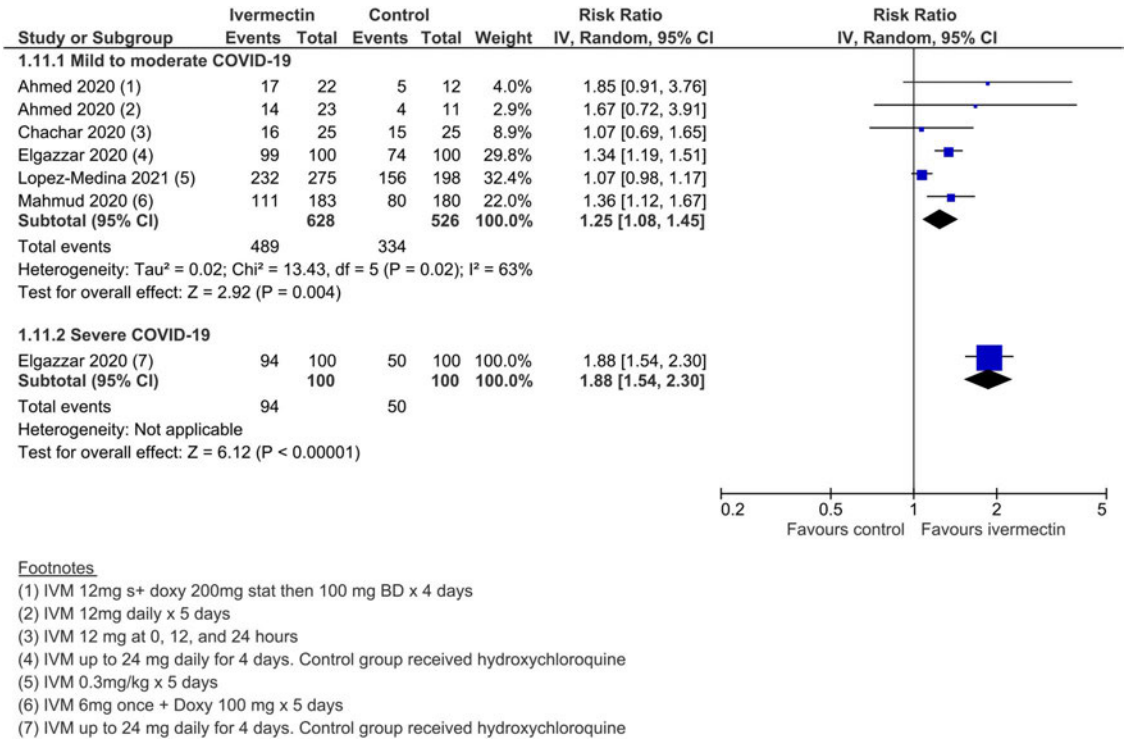


FIGURE 12. Need for mechanical ventilation.



Footnotes
(1) IVM 12mg s+ doxy 200mg stat then 100 mg BD x 4 days
(2) IVM 12mg daily x 5 days
(3) IVM 12 mg at 0, 12, and 24 hours
(4) IVM up to 24 mg daily for 4 days. Control group received hydroxychloroquine
(5) IVM 0.3mg/kg x 5 days
(6) IVM 6mg once + Doxy 100 mg x 5 days
(7) IVM up to 24 mg daily for 4 days. Control group received hydroxychloroquine

FIGURE 13. Improvement.

COVID-19 infection by an average of 86% (79%–91%) (3 trials, 738 participants; aRR 0.14, 95% CI 0.09–0.21; 5.0% vs. 29.6% contracted COVID-19, respectively; *low-certainty evidence*; downgraded due to study design limitations and few included trials) (Figure 15). In 2 trials involving 538 participants, no severe adverse events were recorded (SoF Table 4).

DISCUSSION

The findings indicate with moderate certainty that ivermectin treatment in COVID-19 provides a significant survival benefit. Our certainty of evidence judgment was consolidated by the results of trial sequential analyses, which show that the required IS has probably already been met. Low-certainty evidence on improvement and deterioration also support a likely clinical benefit of ivermectin. Low-certainty evidence suggests a significant effect in prophylaxis. Overall, the evidence also suggests that early use of ivermectin may reduce morbidity and mortality from COVID-19. This is based on (1) reductions in COVID-19 infections when ivermectin was used as prophylaxis, (2) the more favorable effect estimates for mild to moderate disease compared with severe disease for death due to any cause, and (3) on the evidence demonstrating reductions in deterioration.

The evidence on severe adverse events in this review was graded as low certainty, partly because there were too few events to reach statistical significance. Evidence from a recent systematic review of ivermectin use among people with parasitic infections suggests that ivermectin administered at the usual doses (0.2 or 0.4 mg/kg) is safe and could be safe at higher doses.[7,116] A recent World Health Organization document on ivermectin use for scabies found that adverse events with ivermectin were primarily minor and transient.[22]

We restricted the included studies to the highest level of evidence, that is, RCTs, as a policy. This was despite there being numerous observational but nonrandomized trials of ivermectin, which one could argue could also be considered in an emergency. We included preprint and unpublished data from completed but not yet published trials due to the urgency related to evidence synthesis in the context of a global pandemic.[117] Although there is the potential for selective reporting of outcomes and publication bias, we have factored in these considerations in interpreting results and forming conclusions. We adhered to PRISMA guidelines and the WHO statement on developing global norms for sharing data and results during public health emergencies.[117]

There are a number of limitations with this review. Several of the studies contributing data did not

FOI 26-3132 - Document 5

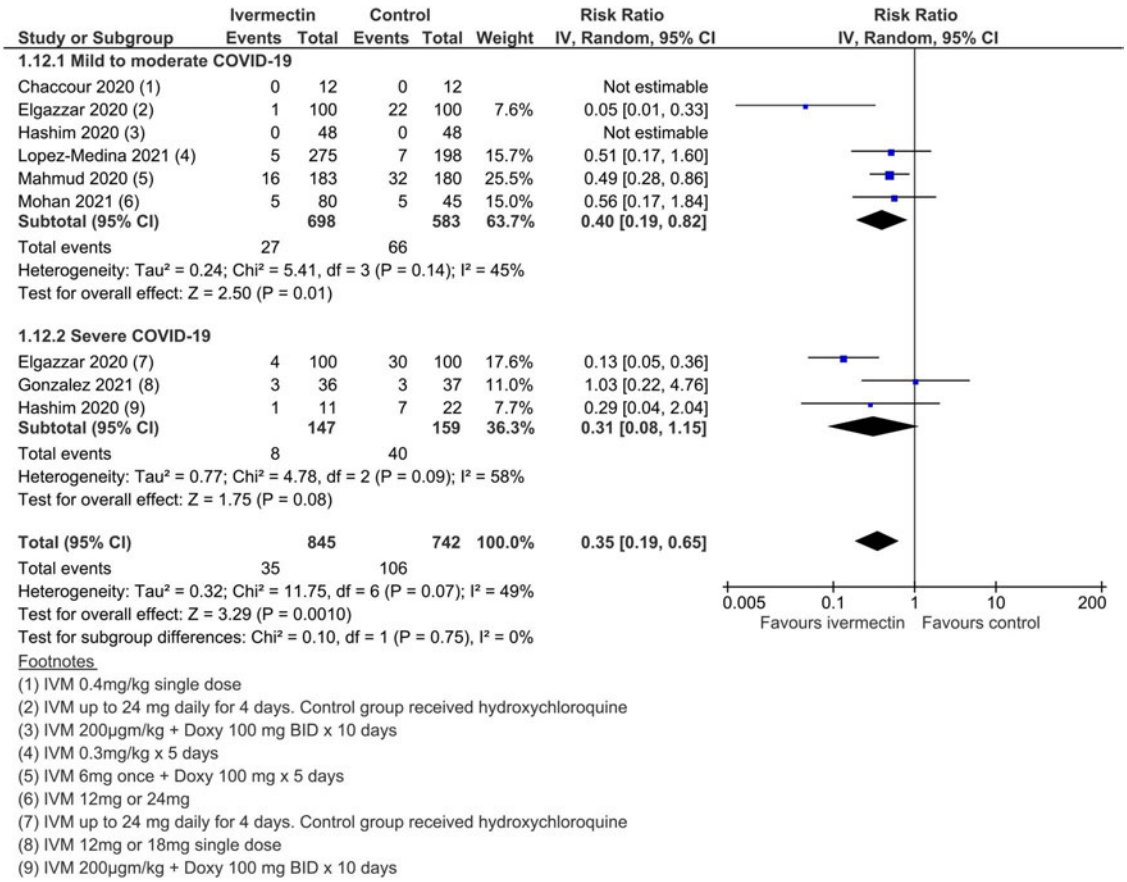


FIGURE 14. Deterioration.

provide full descriptions of methods, so assessing risk of bias was challenging. Where descriptions of study methods were sparse or unclear, we attempted to contact authors to clarify methods, but lack of information led us to downgrade findings in several instances. Overall interpretation of findings was hampered due to variability in the participants recruited, treatment regimen, and the care offered to those in control groups. We have tried to take this variation into account through subgroup and sensitivity analyses. Nevertheless, dosing and treatment regimens and the use of ivermectin with other components of "standard care" require further research. We did not include laboratory outcome measures, such as viral clearance. The latter and other biochemical outcomes have been reported in several studies and reviews and tend to favor ivermectin.[10,47,105,108] Several trials reported continuous data, such as length of hospital stay, as medians and interquartile ranges; therefore, we were unable to include these data in meta-analysis. Because we did not undertake in our protocol to perform narrative evidence synthesis, and because these data tended to favor ivermectin, the certainty of the effects of ivermectin on these continuous outcomes may be underestimated.

At least 5 other reviews of ivermectin use for COVID-19 have been published, including one coauthored with Nobel Laureate Professor Satoshi Ōmura, discoverer of ivermectin,[9,10,118,119,120] but only 3 have been peer-reviewed[9,118,120] and only 2 attempt full systematic review.[10,119] We applied AMSTAR 2,[121] a critical appraisal tool for systematic reviews of health care interventions, to the 2 nonpeered systematic reviews[10,119] and both were judged to be of low quality (Table 5). However, there was also a suggestion that ivermectin reduced the risk of death in treatment of COVID-19 in these reviews.

The recently updated WHO therapeutics guidelines[12] included 7 trials and 1419 people in the analysis of mortality. Reporting a risk reduction of 81% (odds ratio 0.19, 95% CI 0.09–0.36), the effect estimate favoring ivermectin was downgraded by 2 levels for imprecision, although the justification for this is unclear as the reported CI is precise (64%–91%).

In addition to the evidence from systematic reviews, the findings of several controlled observational studies

FOI 26-3132 - Document 5

Table 4. Summary of findings table of ivermectin versus no ivermectin for COVID-19 prophylaxis in healthy population (people without COVID-19 infection).

	Illustrative comparative risks* (95% CI)				
	Assumed risk	Corresponding risk			
Outcomes	No ivermectin	Ivermectin	Relative effect (95% CI)	No of participants (studies)	Quality of the evidence (GRADE)
COVID-19 infection	296 per 1000	245 fewer infections per 1000 (234–269)	RR = 0.14 (0.09–0.21)	738 (3)	Low†
Admission to hospital	Not reported				
Death from any cause	Not reported				
Serious adverse events	No events occurred in 538 participants (2 studies), therefore the effect could not be estimated.				

GRADE working group grades of evidence; High quality: Further research is very unlikely to change our confidence in the estimate of effect; Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate; Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate; Very low quality: We are very uncertain about the estimate.
*The basis for the assumed risk (eg, the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% CI) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).
†Downgraded −2 for study design limitations.
NNT, number needed to treat.

are consistent with existing evidence and suggest improved outcomes with ivermectin treatment.[55,57,59] Similarly, with respect to ivermectin prophylaxis of frontline workers and those at risk, controlled observational studies from Bangladesh and Argentina (the latter which involved 1195 health care workers) have shown apparent reductions in COVID-19 transmission with ivermectin prophylaxis, including in some reports total protection (zero infections) where infection rates in the control group exceeded 50%.[122,123] A very large trial of ivermectin prophylaxis in health care workers in India[124] covered 3532 participants and reported risk ratios not significantly different from this meta-analysis (prophylaxis outcome).

Clarifying ivermectin safety in pregnancy is a key question in patient acceptability for pregnant women contracting COVID-19. A recent meta-analysis[5] found little evidence of increased risk of abnormal pregnancies but similarly weak evidence of absence of risk. For (pre-exposure) prophylaxis in pregnancy, where vaccines may be contraindicated, the alternative of hydroxychloroquine has been advocated.[125,126] In addition to safety and relative efficacy, different risk–benefit judgments may be presented for prophylaxis

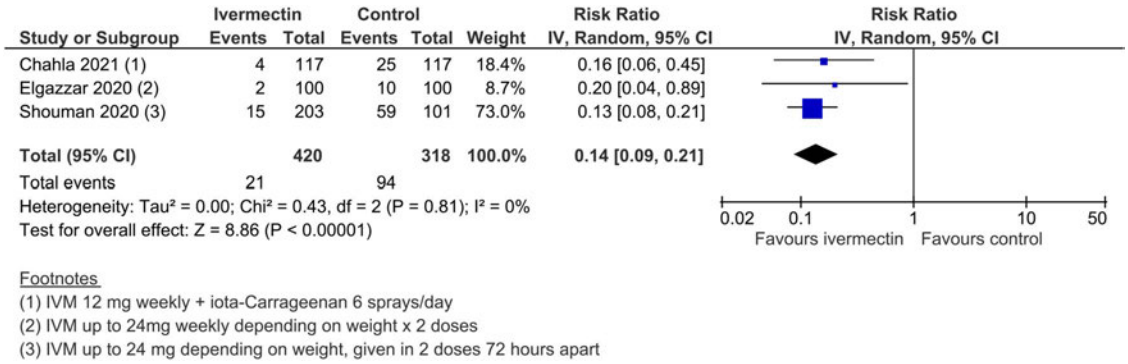


FIGURE 15. COVID-19 infection (prophylaxis studies).

Table 5. Methodological quality of other systematic reviews (AMSTAR 2).

Systematic review	Components of PICO described	A priori study design	Explain selection of study designs	Comprehensive literature search	Duplicate study selection	Duplicate data extraction	List of excluded studies justified	Characteristics of included studies provided
Hill et al, 2021[10]	+	−	+	+	?	?	−*	?†
Castañeda-Sabogal et al 2021[119]	+‖	?	−	?#	+	+	−*	+

Systematic review	Risk of bias adequately assessed and documented	Sources of funding reported	Appropriate methods to combine findings	Appropriate risk-of-bias sensitivity analyses conducted	Risk-of-bias assessment used in conclusions	Satisfactory explanation of observed heterogeneity	Likelihood of publication bias assessed	Conflict of interest stated
Hill et al, 2021[10]	−‡	−	−§	−*	−¶	−*	NA	−
Castañeda-Sabogal et al 2021[119]	−**	−	−††	−‡‡	−*	+	NA	+

Assessed using AMSTAR 2[121]; +, adequately assessed; −, inadequately assessed; ?, unclear assessment; NA, not applicable (less than 10 included studies in meta-analysis).

*Not documented or inadequately reported.

†Participant population, description of comparator interventions, and time frame for follow-up were not described or inadequately reported.

‡No summary of risk-of-bias assessment was given in the main text in the review, other than stating trials were of poor, fair, or high quality. There were some further details about bias in the discussion, but these were largely generic and did not follow the recommended Cochrane tool used to assess risk of bias in RCTs.

§A meta-analysis for all-cause death was presented but authors did not specify why meta-analyses were not conducted for other outcomes, which included at least 2 trials reporting the same comparison and outcome, other than in some parts of the discussion. For example, if viral clearance was reported in most trials, there would have been scope to have performed subgroup analyses and/or split the time point for each comparison to account for the varying duration of follow-up across trials. Instead, they gave a vote count-type narrative of the results, which did not follow synthesis without meta-analysis (SWiM) in systematic review reporting guidelines.[144]

¶There was some further details about bias in the discussion, but this was largely generic and did not follow the recommended Cochrane tool used to assess risk of bias in RCTs. Similarly, in terms of certainty/quality of the evidence, the authors used terms in a summary table that included "good," "fair," and "limited," without offering any explanation or justification.

‖Outcomes were reported but lacked definitions.

#A significant number of pertinent RCTs have not been included in the review. Given the adequate due diligence of review process, the comprehensive nature of the search strategy is questionable.

**No description of risk-of-bias assessment in any domain apart from missing outcome data but attrition rates not documented to justify judgment.

††Authors did not report data from RCTs that we obtained from various sources and some conclusions were not reflective of the observed data. It was reported that in an analysis of 4 preprint retrospective studies at high risk of bias, ivermectin was not associated with reduced mortality (logRR 0.89, 95% CI 0.09–1.70, $P = 0.04$). Although the caveat of studies being at high risk of bias and statistical heterogeneity should be added to any interpretation, it is incorrect to interpret these results as not demonstrating a potential association based on the observed result. Furthermore, the high risk of bias judgment is not adequately justified.

‡‡A sensitivity analysis was performed excluding those studies without adjustment for confounding but no details are provided. Given that there was some evidence of a potential association with ivermectin treatment and survival in 4 retrospective studies (although downplayed as no association due to concerns about attrition), it is highly implausible that any sensitivity analysis would not remove any suggestion of association.

FOI 26-3132 - Document 5

(pre- and post-exposure), and for treatment, with pregnancy a high-risk status for COVID-19.

RCTs in this review did not specifically examine use of ivermectin in the elderly, although this is a known high-risk group for severe COVID-19. In the setting of care homes, it is also notorious for rapid contagion. A standard indication for ivermectin in the elderly is scabies. We identified 2 recent reports suggesting that ivermectin may be efficacious as prevention and treatment of COVID-19 in this age group.[50,127] A letter on positive experience in 7 elder care facilities in Virginia covering 309 patients was sent to NIH[127] and has recently been submitted for publication.

There is also evidence emerging from countries where ivermectin has been implemented. For example, Peru had a very high death toll from COVID-19 early on in the pandemic.[128] Based on observational evidence, the Peruvian government approved ivermectin for use against COVID-19 in May 2020.[128] After implementation, death rates in 8 states were reduced between 64% and 91% over a two-month period.[128] Another analysis of Peruvian data from 24 states with early ivermectin deployment has reported a drop in excess deaths of 59% at 30+ days and of 75% at 45+ days.[129] However, factors such as change in behavior, social distancing, and face-mask use could have played a role in this reduction.

Other considerations related to the use of ivermectin treatment in the COVID-19 pandemic include people's values and preferences, equity implications, acceptability, and feasibility.[130] None of the identified reviews specifically discussed these criteria in relation to ivermectin. However, in health care decision making, evidence on effectiveness is seldom taken in isolation without considering these factors. Ultimately, if ivermectin is to be more widespread in its implementation, then some considerations are needed related to these decision-making criteria specified in the GRADE-DECIDE framework.[130]

There are numerous emerging ongoing clinical trials assessing ivermectin for COVID-19. The trade-off with policy and potential implementation based on evidence synthesis reviews and/or RCTs will vary considerably from country to country. Certain South American countries, Indian states, and, more recently, Slovakia and other countries in Europe have implemented its use for COVID-19.[129,131,132,133,134] A recent survey of global trends[118] documents usage worldwide. Despite ivermectin being a low-cost medication in many countries globally, the apparent shortage of economic evaluations indicates that economic evidence on ivermectin for treatment and prophylaxis of SARS-CoV-2 is currently lacking. This may impact more on LMICs that are potentially waiting for guidance from organizations like the WHO.

Given the evidence of efficacy, safety, low cost, and current death rates, ivermectin is likely to have an impact on health and economic outcomes of the pandemic across many countries. Ivermectin is not a new and experimental drug with an unknown safety profile. It is a WHO "Essential Medicine" already used in several different indications, in colossal cumulative volumes. Corticosteroids have become an accepted standard of care in COVID-19, based on a single RCT of dexamethasone.[1] If a single RCT is sufficient for the adoption of dexamethasone, then a fortiori the evidence of 2 dozen RCTs supports the adoption of ivermectin.

Ivermectin is likely to be an equitable, acceptable, and feasible global intervention against COVID-19. Health professionals should strongly consider its use, in both treatment and prophylaxis.

ACKNOWLEDGMENTS

This work was inspired by the prior literature review of Dr Pierre Kory.

The authors thank Information Specialist, Jo Platt, of the Cochrane Gynaecological, Neuro-oncology, and Orphan Cancer (CGNOC) group for designing the search strategy and running the search, as well as Anna Noel Storr for reviewing the strategy. The authors also thank Isabella Rushforth for her voluntary assistance with the preparation of the reference lists.

The authors thank Gill Gyte for detailed comments, feedback, and involvement in this review, and Michael Grayling and David Tovey for useful peer review comments before submission. The authors also thank the external peer reviewers for their helpful comments and Peter Manu for the opportunity to publish our findings.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

International Journal of Antimicrobial Agents 59 (2022) 106516

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

High-dose ivermectin for early treatment of COVID-19 (COVER study): a randomised, double-blind, multicentre, phase II, dose-finding, proof-of-concept clinical trial

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ARTICLE INFO

Article history:
Received 9 November 2021
Accepted 22 December 2021

Editor Dr Jim Gray

Keywords:
Ivermectin
SARS-CoV-2
COVID-19
Randomised controlled trial

ABSTRACT

High concentrations of ivermectin demonstrated antiviral activity against SARS-CoV-2 in vitro. The aim of this study was to assess the safety and efficacy of high-dose ivermectin in reducing viral load in individuals with early SARS-CoV-2 infection. This was a randomised, double-blind, multicentre, phase II, dose-finding, proof-of-concept clinical trial. Participants were adults recently diagnosed with asymptomatic/oligosymptomatic SARS-CoV-2 infection. Exclusion criteria were: pregnant or lactating women; CNS disease; dialysis; severe medical condition with prognosis <6 months; warfarin treatment; and antiviral/chloroquine phosphate/hydroxychloroquine treatment. Participants were assigned (ratio 1:1:1) according to a randomised permuted block procedure to one of the following arms: placebo (arm A); single-dose ivermectin 600 μg/kg plus placebo for 5 days (arm B); and single-dose ivermectin 1200 μg/kg for 5 days (arm C). Primary outcomes were serious adverse drug reactions (SADRs) and change in viral load at Day 7. From 31 July 2020 to 26 May 2021, 32 participants were randomised to arm A, 29 to arm B and 32 to arm C. Recruitment was stopped on 10 June because of a dramatic drop in cases. The safety analysis included 89 participants and the change in viral load was calculated in 87 participants. No SADRs were registered. Mean (S.D.) $\log_{10}$ viral load reduction was 2.9 (1.6) in arm C, 2.5 (2.2) in arm B and 2.0 (2.1) in arm A, with no significant differences ($P = 0.099$ and 0.122 for C vs. A and B vs. A, respectively). High-dose ivermectin was safe but did not show efficacy to reduce viral load.

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1. Introduction

The vaccine roll-out has certainly played a key role in the fight against COVID-19 (coronavirus disease 2019). However, the emergence of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) variants and the (slow) pace of vaccination have

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https://doi.org/10.1016/j.ijantimicag.2021.106516
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FOI 26-3132 - Document 5

hampered the expected drop in cases in most parts of the world [1,2]. Thus, the pandemic is still hitting hard and antiviral drugs, preferably at low cost and widely available, are still badly needed.

Initially, research has been oriented towards drug repurposing, which is time-saving compared with new drug discovery [3]. Several compounds have been tested against SARS-CoV-2 in vitro and subsequently in vivo, including drugs not primarily used as antiviral agents. In particular, chloroquine/hydroxychloroquine and ivermectin were of interest for use in low- and middle-income countries as these drugs are widely available (for treatment of parasitic infections) and inexpensive [3]. Although recent evidence has globally shifted attention towards novel (and much more expensive) ad hoc medications [4–7], interest in a possible role for cheaper and older compounds remains.

In March 2020, Australian researchers demonstrated that ivermectin, a drug used for decades to combat parasitic infections, had antiviral activity against SARS-CoV-2 in vitro in Vero cell cultures, virtually reducing the viral load to zero in 48 h [8]. This sparked considerable interest in this 'old' drug as a potential cure for COVID-19, resulting on one hand in a consistent number of clinical trials testing ivermectin efficacy, and on the other in an uncontrolled procurement of the drug for self-treatment [9]. However, a plasma concentration of ivermectin compatible with the IC_{50} (half maximal inhibitory concentration) found in vitro (~2.5 μM, equivalent to 2190 ng/mL) is far from being achievable with the usual doses of this drug [10] (40–80 ng/mL after a dose of 200–400 μg/kg). Based on this, we would expect ivermectin to show neither clinical nor virological efficacy against COVID-19 unless higher doses are administered.

The aim of this study was to assess the efficacy and safety of high doses of ivermectin (namely doses of 600 μg/kg and 1200 μg/kg for 5 consecutive days) for the treatment of SARS-CoV-2 infection. The doses were chosen based on the following considerations: (i) according to the reported pharmacokinetic (PK) profile in plasma [11,12], it can be supposed that repeated daily administrations result in drug accumulation, with predicted plasma levels only slightly higher than those observed with single administration of 2000 μg/kg in fasted state (248 ng/mL), which was well tolerated [11]; and (ii) studies in mammals [13,14] showed much higher ivermectin levels in pulmonary tissue than in plasma.

The primary objectives of this study were to define: (i) whether ivermectin, administered at two different high dosages, is safe in participants with initial, asymptomatic or oligosymptomatic SARS-CoV-2 infection; and (ii) whether ivermectin, administered at the dosage(s) found to be safe decreases the viral load of SARS-CoV-2 at Day 7.

2. Materials and methods

2.1. Study design

The COVER study was a randomised, investigator-initiated, double-blind, multicentre, phase II, dose-finding, proof-of-concept clinical trial carried out in outpatients consecutively diagnosed in four sites in Italy. The study protocol (available in the Supplementary material) was approved by the national Ethics Committee of INMI–Spallanzani in Rome, which is competent for all COVID-19 trials in Italy (resolution 139/2020 of 28 May 2020) and by the Italian drug agency AIFA (resolution 136BIS/2020 of 18 May 2020). The protocol was also registered with ClinicalTrials.gov (NCT04438850). The study complied with the Declaration of Helsinki and was performed in accordance with Good Clinical Practice guidelines. All participants provided written informed consent.

2.2. Participants and study sites

Participants were adult (≥18 years) subjects newly diagnosed with SARS-CoV-2 infection by real-time PCR analysis of nasopharyngeal swabs, not requiring hospitalisation or oxygen supplementation (COVID-19 severity score <3 [15]) and providing their informed consent to the study. The main exclusion criteria were: pregnant or lactating women; known central nervous system disease; participants receiving dialysis; any severe medical condition with a prognosis of <6 months; warfarin treatment; antiviral treatment; and chloroquine phosphate or hydroxychloroquine treatment.

2.3. Randomisation and masking

The study arms were as follows: placebo arm (arm A); single-dose ivermectin 600 μg/kg plus placebo for 5 days (arm B); and single-dose ivermectin 1200 μg/kg for 5 days (arm C).

Participants were randomly assigned by a centralised computer system to one of the three arms with an allocation ratio of 1:1:1. The study biostatistician prepared the sequence of treatments, generated using SAS 9.4 software according to a randomised permuted blocks procedure. The treatment ID was obtained through REDCap, used as a web-based clinical data management system for the study. Following randomisation, the treatment ID and the patient's weight were communicated to the hospital pharmacist who was in charge to prepare the study treatment according to the randomisation list. In order to keep investigators and participants blinded, the number of tablets to be administered was the same, irrespective of the study arm in which the participant was randomised. Placebo tablets were identical in appearance and taste to ivermectin. Also, the staff in charge of the laboratory analyses were blinded.

Following randomisation, participants were provided the daily therapy in five packs, numbered 1 to 5 with the respective dates and labelled with the randomisation code. The division into single doses and the labelling were done by the study pharmacists under controlled conditions. The daily doses were self-administered on an empty stomach with water.

2.4. Study procedures

Starting with Day 1 (day of first dose), investigators contacted the participants daily by phone until Day 5 in order to check for the correct intake of the drug and for the occurrence of any adverse events (AEs) based on a pre-specified list. At Day 7, the participant was re-assessed at an in-person visit and a nasopharyngeal swab was performed and examined locally by RT-PCR, then stored at –80°C until the final (centralised) analysis. Full blood count and transaminases were also checked locally. Further onsite visits were scheduled on Day 14 (in all similar to Day 7) and Day 30 (final follow-up visit). Nasopharyngeal swabs were performed if positive at previous visit, and the leftover material was stored as above. Unscheduled visits for any reason were also foreseen. Participants were provided with a dedicated phone number so that they could contact the investigators in case of need.

AEs were assessed for severity according to National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE) grading v.4.03, and the causal relationship to the study treatment or to SARS-CoV-2 infection was established by the study physician and recorded in the electronic case report form (eCRF).

Serious adverse events (SAEs) or serious adverse drug reactions (SADRs) had to be immediately (not later than 24 h) reported to the sponsor as well as to the Study Safety Desk. The same applied to suspected unexpected serious adverse reactions (SUSARs).

FOI 26-3132 - Document 5

2.5. Study outcomes

Primary outcomes were: (i) number of SADRs; and (ii) change in viral load at Day 7 with respect to baseline.

Secondary outcomes were: (i) trend over time in quantitative viral load at Days 7, 14 and 30; (ii) time to clinical resolution (TCR) (if symptomatic); (iii) proportion of participants with virological clearance at Days 14 and 30; (iv) hospitalisation rate; and (v) COVID-19 severity score at Days 14 and 30.

2.6. Statistical analysis

The statistical study design was conceived in two steps.

Step 1 aimed at testing the safety of the two experimental arms, in terms of SADRs occurrence, on the first 60 evaluable participants (20 per arm). If the experimental arm failed to be demonstrated as safe, the allocation of participants was planned to be interrupted. At the same time, interim analyses on efficacy and futility were planned comparing control and experimental arms. Futility and efficacy criteria for prematurely stopping the trial are detailed in the study protocol.

Step 2 aimed at testing the efficacy of experimental arms, considered safe at first stage, compared with the control arm in terms of viral load decrease.

The primary safety endpoint was the occurrence of a SADR. According to A'Hern's [16] single-stage design, setting a type I error rate of 10% and power of 80% and hypothesising that the true toxicity rate (SADRs) was 5%, 20 participants in each experimental arm were needed to test the null hypothesis that the toxicity rate was 30% against a one-sided alternative.

The primary efficacy endpoint was the difference in viral load decline from baseline to Day 7. Viral load was measured as $\log_{10}$ of genome copies per μL, ascertained by droplet digital quantitative PCR (dd-PCR) [17,18]. To et al. [19] reported a difference in mean viral load decline of 1.05 $\log_{10}$ copies [standard deviation (S.D.) 0.69 $\log_{10}$ copies]. The desired difference in decrease between each experimental group and control was at least 0.47 $\log_{10}$ copies/mL (effect size $\Delta = 0.68$, considered to be of moderate-large magnitude according to Cohen [20]). According to these hypotheses, with 34 participants per arm the study had a power of 80% to detect a difference between control and experimental arms, at 0.025 α level, one-sided. Therefore, if both experimental treatments proceeded to step 2 and were compared with the control group, the total number of participants to be analysed had to be 102. Considering a potential 20% loss to follow-up, including missing or inadequate specimens, it was planned to enrol 129 participants.

Data were analysed according to the Statistical Analysis Plan (Supplementary material) using SAS 9.4 software. The primary safety analysis was performed on the safety analysis set including participants who received at least one dose of study treatment.

Analysis of the primary efficacy endpoint was performed on the evaluable analysis set, considering all participants as originally assigned to the treatment arms without major violations of eligibility criteria and having the viral load evaluable at Day 7.

A per-protocol (PP) analysis set was defined including only participants who took the allocated treatment as specified in the protocol for 5 days in order to check for consistency with the primary analysis.

2.6.1. Analysis of primary outcomes

The proportion of participants in each experimental group experiencing at least one SADR was described by means of frequency and percentage.

The mean of the differences in viral load decline from baseline to Day 7 between each experimental group and the control group was described by standard summary measures for continuous data and was compared by Student's *T*-test and Wilcoxon test (according to Shapiro–Wilk test for normality).

2.6.2. Analysis of secondary outcomes

A full description of the statistical analyses of all secondary outcomes is reported in the Statistical Analysis Plan (Supplementary material). Assessment of the tolerability profile was mainly based on adverse drug reactions and the frequency and nature of SAEs. The proportion of participants in each group experiencing AEs was compared by χ^2 test (or Fisher's exact test when appropriate). Generalised longitudinal mixed models were used to analyse the trend over time in viral load at the different time points. TCR was calculated as the time from randomisation to clinical resolution or death. TCR was analysed with Cox regression models and was described by the Kaplan–Meier method. The proportion of participants with virological clearance was described by frequencies and proportions with their relative exact 95% confidence interval (CI). The χ^2 test was used to test the proportion of participants with virological clearance at Day 14, and Fisher's exact test was used at Day 30.

2.6.3. Ancillary study on pharmacokinetics

Measurement of plasma concentrations of ivermectin was included as an ancillary study to inform on the maximal drug levels and drug accumulation following repeated doses, on the interindividual variability and, possibly, on the association between blood drug concentration and clinical endpoints. In fact, a high variability in absorption has been described [11]. Peak plasma concentrations (C_{max}) are observed at approximately 4 h after dosing, with an elimination half-life of 18–35 h [11,12]. For these reasons and for feasibility considerations, it was planned to collect blood samples in a subset of participants at three timepoints: just before and 4 h and 48 h after the fifth dose. Plasma aliquots were stored at –20°C (or lower temperature) until analysis.

Ivermectin was measured by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) at the laboratory of the IRCCS Sacro Cuore Don Calabria Hospital using a method validated according to the European Medicines Agency (EMA) guidelines. Details on the procedure are reported in the study protocol.

2.7. Role of the funding sources

The sponsor was IRCCS Sacro Cuore Don Calabria Hospital, which received funds for this trial from the Italian Ministry of Health in the framework of 'Ricerca corrente'. Tablets of 9 mg ivermectin and placebo were donated by Insud Pharma (Madrid, Spain). The funders had no role in study design, data collection, analysis and interpretation, or writing of the report.

3. Results

From 31 July 2020 to 26 May 2021, 93 participants were randomised to the three study arms: 32 to arm A; 29 to arm B; and 32 to arm C. IRCCS Ospedale Sacro Cuore Don Calabria of Negrar contributed 79 participants, Ospedale 'Luigi Sacco' ASST Fatebenefratelli Sacco of Milano contributed 8 participants, Azienda Ospedaliera-Universitaria–Policlinico S. Orsola-Malpighi of Bologna contributed 4 participants and Azienda Provinciale Servizi Sanitari Trento–Ospedale of Rovereto contributed 2 participants.

In March 2021, at the interim analysis performed after the enrolment of 64 participants, no SADRs were observed. Furthermore, none of the experimental arms were stopped for efficacy or futility.

Recruitment was stopped on 10 June 2021 because of a dramatic drop in cases. The last follow-up visit was carried out on 29 June 2021.

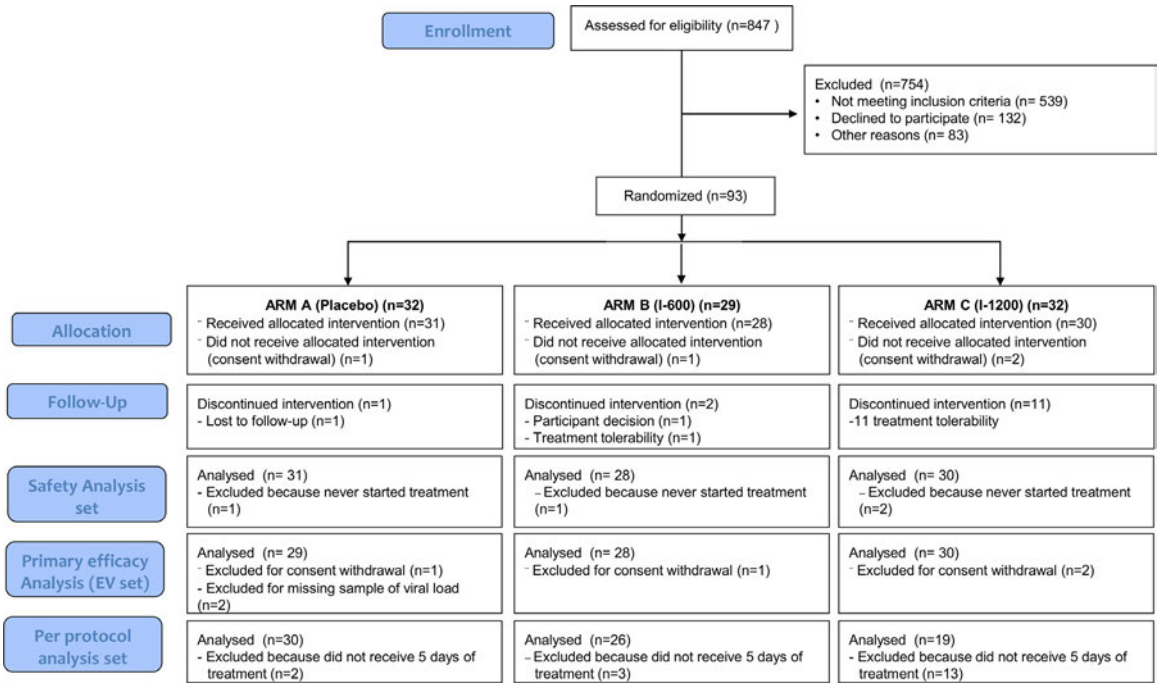


Fig. 1. Study flow chart. Information regarding arm of randomisation, number of subjects screened and enrolled, and detailed reasons for missing inclusion are presented.

Detailed information on screened participants and the reasons for missing inclusions were retrieved from the screening log handled by the centres and is summarised in the study flow chart (Fig. 1; Supplementary Table S1). This information was provided by all centres but one (Rovereto, which contributed two randomised participants). Among the other centres, 845 participants were screened and 91 (10.8%) were randomised. The main reasons for exclusion were infection not recent (56.9%), patient refusal (15.4%), severity score >2 (7.8%) and organisational reasons (7.2%). The 93 participants were randomly assigned to the treatment arms. Of the 93 participants, 4 never started the treatment and withdrew consent and the other 89 received at least 1 day of study treatment and were therefore included in the analysis of the primary endpoints. Moreover, 2 participants (both in arm A) had a missing sample for viral load at Day 7, therefore 87 participants (93.5%) were included in the primary endpoint for efficacy. Moreover, 75 participants (80.6%) received 5 days of treatment and were included in the PP analysis set.

The main baseline demographic and clinical characteristics of the study population are summarised in Table 1 for all randomised participants and in Supplementary Table S2 for participants included in the evaluable analysis set. Male sex was more represented (58.1%). The median age was 47.0 years [interquartile range (IQR) 31.0–58.0]. The majority of participants were European (96.8%). Co-morbidities were reported by one-third of participants. Eighty participants (86.0%) were symptomatic with a median duration of 4 days, with cough being the most frequent symptom, followed by fever and fatigue. The severity score was 1 for 78 participants (83.9%) and 2 for 15 participants (16.1%).

Details of the main physical findings, baseline laboratory examinations and concomitant treatments are summarised in Supplementary Tables S3–S5.

Summary of treatment compliance is reported in Table 2. Fourteen participants (15.1%) discontinued treatment: 1 (3.1%) in arm A; 2 (6.9%) in arm B; and 11 (34.4%) in arm C. The interruptions in arm C were all due to tolerability. Seven participants received only 1 day of treatment, three participants received 2 days and 3 days, respectively, and one participant received 4 days (Supplementary Table S6).

No SADRs were observed in any of the study groups. Results for viral load ($\log_{10}$) at Day 7 versus baseline are summarised in Table 3. The mean reduction in viral load was 2.9 (SD 1.6) in arm C versus 2.5 (SD 2.2) in arm B and 2.0 (SD 2.1) in arm A. The observed effect size (versus arm A) was 0.48 for arm C and 0.21 for arm B. Differences were not normally distributed (Shapiro–Wilk test P-value <0.0001 for both comparisons), thus Wilcoxon exact test was also performed. The differences were not significant ($P = 0.099$ and 0.122 for arm C vs. arm A and arm B vs. arm A, respectively). Results according to PP analysis, summarised in Supplementary Table S7, are superimposable.

The trend over time of the quantitative viral load is summarised in Supplementary Tables S8 and S9. In both non-adjusted and multivariate mixed model, a significant interaction was found between arm C and Day 7 (P-value = 0.035 and 0.036, respectively). TCR was described in symptomatic participants (80 participants). The median TCR was 14 days (IQR 13–30 days), 29 days (IQR 13.5–32.0 days) and 14 days (IQR 7–37 days) for arms A, B and C, respectively. Kaplan–Meier curves for TCR comparisons between arms are reported in Supplementary Fig. S1. Differences between arms were not significant [hazard ratio (HR) = 0.69, 95% CI 0.36–1.32 ($P = 0.262$) and HR = 0.79, 95% CI 0.42–1.47 ($P = 0.456$) for arm B vs. arm A and for arm C vs. arm A, respectively] (Supplementary Table S10). The proportion of participants with virological clearance at Days 14 and 30 is summarised in Supplementary Table S11. Within 14 days, 59% (95% CI 22.4–61.2%), 70% (95% CI 13.2–52.9%) and 58% (95% CI 23.4–63.1%) of participants in arms A, B and C achieved virological clearance.

As an exploratory analysis, the same proportion for Day 7 was also added. No significant difference between arms was observed at any of the time points. The hospitalisation rate was 3/30 (10.0%) for arm C, 1/29 (3.4%) for arm B and 0 for arm A. Regarding the COVID-19 severity score, at Day 14 all participants had a score of 1, except for two participants in arm B with severity scores of 2 and 3. At Day 30 all participants had a severity score of 1.

Table 1
Demographic and baseline characteristics of the study participants, overall and by study arm

Characteristic	Arm A (N = 32)	Arm B (N = 29)	Arm C (N = 32)	Overall (N = 93)
Age (years) [median (IQR)]	50.0 (26.0–57.0)	47.0 (31.0–62.0)	44.5 (31.0–55.5)	47.0 (31.0–58.0)
Female sex [n (%)]	17 (53.1)	14 (48.3)	8 (25.0)	39 (41.9)
Weight (kg) [median (IQR)]	69.0 (62.5–74.0)	72.0 (61.0–84.0)	79.0 (70.5–85.0)	72.0 (63.0–82.0)
Height (cm) [median (IQR)]	170.0 (164.5–178.0)	170.0 (167.0–175.0)	173.0 (170.0–180.0)	170.0 (167.0–178.0)
Nation of origin [n (%)]				
European	29 (90.6)	29 (100.0)	32 (100.0)	90 (96.8)
Extra-European	3 (9.4)	0 (0.0)	0 (0.0)	3 (3.2)
Setting of baseline visit [n (%)]				
Home	27 (84.4)	24 (82.8)	23 (71.9)	74 (79.6)
Hospital emergency room	3 (9.4)	2 (6.9)	6 (18.8)	11 (11.8)
Hospital outpatient ambulatory care	1 (3.1)	2 (6.9)	3 (9.4)	6 (6.5)
Other	1 (3.1)	1 (3.4)	0 (0.0)	2 (2.2)
Co-morbidities [n (%)]	8 (25.0)	11 (37.9)	12 (37.5)	31 (33.3)
Respiratory	0 (0.0)	4 (36.4)	2 (16.7)	6 (19.4)
Cardiovascular	7 (87.5)	7 (63.6)	8 (66.7)	22 (71.0)
Diabetes	2 (25.0)	0 (0.0)	1 (8.3)	3 (9.7)
Time from diagnosis to randomisation (days) [median (IQR)]	1.0 (0.0–1.0)	1.0 (1.0–2.0)	1.0 (0.5–2.0)	1.0 (1.0–2.0)
Symptoms [n (%)]	27 (84.4)	24 (82.8)	29 (90.6)	80 (86.0)
Cough	10 (37.0)	9 (37.5)	16 (55.2)	35 (43.8)
Pyrexia (>37.5°C)	9 (33.3)	8 (33.3)	16 (55.2)	33 (41.3)
Fatigue	10 (37.0)	6 (25.0)	10 (34.5)	26 (32.5)
Myalgia	6 (22.2)	3 (12.5)	13 (44.8)	22 (27.5)
Headache	7 (25.9)	4 (16.7)	9 (31.0)	20 (25.0)
Anosmia	4 (14.8)	4 (16.7)	9 (31.0)	17 (21.3)
Infective rhinitis	4 (14.8)	10 (41.7)	1 (3.4)	15 (18.8)
Dysgeusia	4 (14.8)	2 (8.3)	2 (6.9)	8 (10.0)
Oropharyngeal pain	2 (7.4)	4 (16.7)	3 (10.3)	9 (11.3)
Diarrhoea	0 (0.0)	3 (12.5)	2 (6.9)	5 (6.3)
Asthenia	3 (11.1)	0 (0.0)	2 (6.9)	5 (6.3)
Other [a]	7 (25.9)	5 (20.8)	3 (10.3)	15 (18.8)
Days with symptoms [median (IQR)]	4.0 (2.0–6.0)	4.0 (3.0–5.0)	4.0 (3.0–6.0)	4.0 (3.0–5.5)
COVID-19 severity score [n (%)]				
1, no limitation of activities	27 (84.4)	24 (82.8)	27 (84.4)	78 (83.9)
2, limitation of activities	5 (15.6)	5 (17.2)	5 (15.6)	15 (16.1)
SARS CoV-2 vaccine [n (%)]	1 (3.1)	1 (3.4)	0 (0.0)	2 (2.2)

IQR, interquartile range; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
NOTE: Arm A, placebo; arm B, ivermectin 600 μg/kg + placebo for 5 days; and arm C, ivermectin 1200 μg/kg for 5 days.
[a] Includes back pain, arthralgia, nausea, odynophagia, dyspnoea, ageusia, decreased appetite, ear pain, hyperaesthesia, musculoskeletal pain, pharyngitis and pruritic rash.

Table 2
Summary of treatment compliance, overall and by study arm

	Arm A (N = 32)	Arm B (N = 29)	Arm C (N = 32)	Overall (N = 93)
Treatment never started [n (%)]	1 (3.1)	1 (3.4)	2 (6.3)	4 (4.3)
Reason treatment for never started [n (%)]				
Consent withdrawal	1 (100.0)	1 (100.0)	2 (100.0)	4 (100.0)
Treatment discontinued [n (%)]	1 (3.1)	2 (6.9)	11 (34.4)	14 (15.1)
Reason for discontinuation [n (%)]				
Treatment tolerability	0 (0.0)	1 (50.0)	11 (100.0)	12 (85.7)
Participant decision	0 (0.0)	1 (50.0)	0 (0.0)	1 (7.1)
Lost to follow-up	1 (100.0)	0 (0.0)	0 (0.0)	1 (7.1)
Treatment completed [n (%)]	30 (93.8)	26 (89.7)	19 (59.4)	75 (80.6)

NOTE: Arm A, placebo; arm B, ivermectin 600 μg/kg + placebo for 5 days; and arm C, ivermectin 1200 μg/kg for 5 days.

The maximum grade of each AE per participant is summarised in Table 4 and in Supplementary Table S12. Overall, 229 AEs were recorded, 46 in arm A (20.1%), 69 in arm B (30.1%) and 114 in Arm C (49.8%). Only two AEs were grade 3 and no AEs of grades 4 or 5 were observed. The most reported AE concerned transient eye disorders [21/30 participants (70.0%) in arm C, 13/28 participants (46.4%) in arm B and 1/31 participants (3.2%) in arm A], followed by nervous system disorders, fatigue and gastrointestinal symptoms.

Four AEs were recorded as serious (SAEs): in all cases they required hospitalisation for worsening of the disease with no causal relationship to the study drug. Three SAEs occurred in arm C, 1 in arm B and none in arm A. All events resolved.

3.1. Ancillary study on pharmacokinetics

Concentrations of ivermectin were measured, in blind, in the plasma of 15 participants. The results are summarised in Fig. 2. The drug was not detectable in the plasma of five participants of arm A (placebo). Seven participants belonged to arm B and three to arm C, and in two of the latter the third time point was 72 h after the fifth dose. At T4 (4 h after the fifth dose), i.e. the time corresponding to the published C_{max}, the concentration ranged from 79.2 to 414.8 ng/mL for arm B and from 337.1 to 1082.0 ng/mL for arm C. The area under the concentration–time curve (AUC), calculated for the observed 0–48 h period (that is AUC_{96-144} if taking into account that measurements were done after the fifth dose)

FOI 26-3132 - Document 5

Table 3
Primary efficacy endpoint of viral load in the evaluable analysis set, overall and by study arm

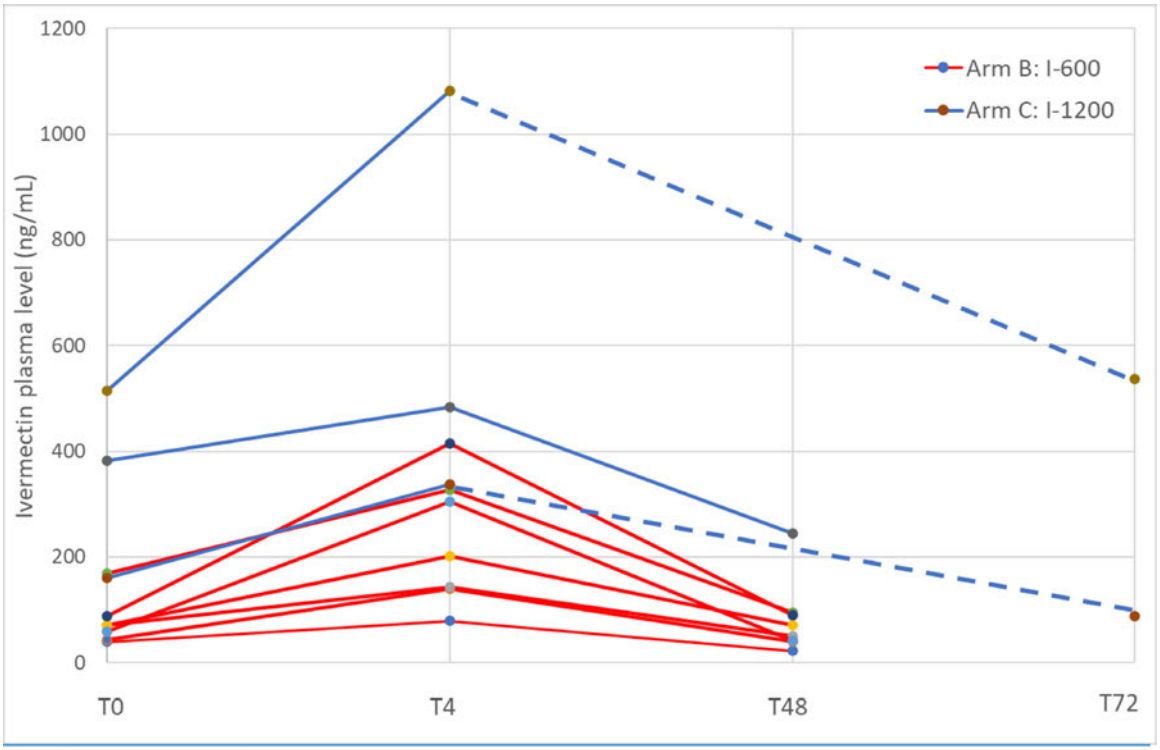
	Arm A ($N = 29$)	Arm B ($N = 28$)	Arm C ($N = 30$)	Overall ($N = 87$)
SARS-CoV-2 viral load at baseline (log_{10})				
Mean ± S.D.	4.3 ± 1.3	4.3 ± 1.2	4.5 ± 1.2	4.4 ± 1.2
Median (IQR)	4.4 (3.5–5.2)	4.3 (3.8–5.1)	4.4 (4.0–5.2)	4.4 (3.8–5.2)
Range	1.7–6.6	2.3–6.6	2.1–7.0	1.7–7.0
Missing	1	0	1	2
SARS-CoV-2 viral load at Day 7 (log_{10})				
Mean ± S.D.	2.2 ± 1.5	1.9 ± 1.6	1.6 ± 1.2	1.9 ± 1.4
Median (IQR)	2.3 (1.1–2.8)	1.5 (0.9–2.5)	1.6 (0.9–2.5)	1.7 (0.9–2.7)
Range	0.0–6.7	0.0–5.2	0.0–5.4	0.0–6.7
Differences in viral load decline from baseline to 7 days				
Mean ± S.D.	2.0 ± 2.1	2.5 ± 2.2	2.9 ± 1.6	2.5 ± 2.0
Median (IQR)	2.6 (1.6–3.2)	3.1 (2.3–4.1)	3.1 (1.8–4.1)	2.8 (1.7–3.7)
Range	–4.8 to 4.9	–2.9 to 4.9	–0.2 to 5.5	–4.8 to 5.5
Missing	1	0	1	2
Effect size				
Arm B vs. arm A				0.21
Arm C vs. arm A				0.48
Shapiro–Wilk test *P*-value (test for normal distribution)				
Arm B vs. arm A				<0.0001
Arm C vs. arm A				<0.0001
Wilcoxon exact *P*-value				
Arm B vs. arm A				0.122
Arm C vs. arm A				0.099
T-test *P*-value				
Arm B vs. arm A				0.429
Arm C vs. arm A				0.078

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; S.D., standard deviation; IQR, interquartile range.
NOTE: Arm A, placebo; arm B, ivermectin 600 μg/kg + placebo for 5 days; and arm C, ivermectin 1200 μg/kg for 5 days.

Table 4
Adverse events in the safety analysis set

Adverse event/arm	Adverse events [*n* (%)] Grade 0	Grade 1	Grade 2	Grade 3	χ^2 for trend arm B vs. arm A	χ^2 for trend arm C vs. arm A
Eye disorders [a]					<0.0001	<0.0001
Arm A ($N = 31$)	30 (96.8)	1 (3.2)	0 (0.0)	0 (0.0)		
Arm B ($N = 28$)	15 (53.6)	12 (42.9)	1 (3.6)	0 (0.0)		
Arm C ($N = 30$)	9 (30.0)	16 (53.3)	5 (16.7)	0 (0.0)		
Gastrointestinal disorders [b]					0.506	0.029
Arm A ($N = 31$)	25 (80.6)	5 (16.1)	1 (3.2)	0 (0.0)		
Arm B ($N = 28$)	21 (75.0)	5 (17.9)	2 (7.1)	0 (0.0)		
Arm C ($N = 30$)	17 (56.7)	8 (26.7)	5 (16.7)	0 (0.0)		
General disorders and administration site conditions [c]					0.185	0.023
Arm A ($N = 31$)	17 (54.8)	13 (41.9)	1 (3.2)	0 (0.0)		
Arm B ($N = 28$)	10 (35.7)	17 (60.7)	1 (3.6)	0 (0.0)		
Arm C ($N = 30$)	8 (26.7)	19 (63.3)	3 (10.0)	0 (0.0)		
Infections and infestations [d]					0.293	0.080
Arm A ($N = 31$)	31 (100)	0 (0.0)	0 (0.0)	0 (0.0)		
Arm B ($N = 28$)	27 (96.4)	0 (0.0)	0 (0.0)	1 (3.6)		
Arm C ($N = 30$)	27 (90.0)	0 (0.0)	2 (6.7)	1 (3.3)		
Musculoskeletal and connective tissue disorders [e]					0.293	-
Arm A ($N = 31$)	31 (100)	0 (0.0)	0 (0.0)	0 (0.0)		
Arm B ($N = 28$)	27 (96.4)	1 (3.6)	0 (0.0)	0 (0.0)		
Arm C ($N = 30$)	30 (100)	0 (0.0)	0 (0.0)	0 (0.0)		
Nervous system disorders [f]					0.711	0.091
Arm A ($N = 31$)	16 (51.6)	14 (45.2)	1 (3.2)	0 (0.0)		
Arm B ($N = 28$)	15 (53.6)	13 (46.4)	0 (0.0)	0 (0.0)		
Arm C ($N = 30$)	11 (36.7)	14 (46.7)	5 (16.7)	0 (0.0)		
Vascular disorders [g]					0.293	0.147
Arm A ($N = 31$)	31 (100)	0 (0.0)	0 (0.0)	0 (0.0)		
Arm B ($N = 28$)	27 (96.4)	0 (0.0)	1 (3.6)	0 (0.0)		
Arm C ($N = 30$)	28 (93.3)	0 (0.0)	2 (6.7)	0 (0.0)		

NOTE: Arm A, placebo; arm B, ivermectin 600 μg/kg + placebo for 5 days; and arm C, ivermectin 1200 μg/kg for 5 days.
[a] Includes photophobia, photopsia, blurred vision, visual impairment and vitreous floaters.
[b] Includes abdominal pain, diarrhoea, nausea and vomiting.
[c] Includes fatigue, gait disturbance and malaise.
[d] Includes COVID-19 (coronavirus disease 2019) pneumonia.
[e] Includes arthralgia.
[f] Includes dizziness, headache, paraesthesia and somnolence.
[g] Includes hypotension.



Key	Arm	T0	T4	T48	T72	AUC
768-1	Arm A: placebo	0	0	0		0
768-5	Arm A: placebo	0	0	0		0
776-3	Arm A: placebo	0	0	0		0
776-6	Arm A: placebo	0	0	0		0
776-9	Arm A: placebo	0	0	0		0
776-63	Arm B: I-600	39.9	79.2	22.6		2478
776-1	Arm B: I-600	44.1	140.5	40.9		4360
776-65	Arm B: I-600	71.5	142.6	51.1		4690
776-74	Arm B: I-600	70.9	202.4	70.4		6548
776-4	Arm B: I-600	58.3	305.1	42.3		8370
776-54	Arm B: I-600	169.5	327.3	95.5		10295
776-7	Arm B: I-600	88.6	414.8	89.3		12097
776-2	Arm C: I-1200	161.1	337.1		88.6	13304
768-2	Arm C: I-1200	381.9	483.3	245.3		17760
776-11	Arm C: I-1200	514.3	1082		537.4	50096

Fig. 2. Pharmacokinetic results for ivermectin concentrations in 15 participants (Key). The drug concentration was measured at baseline (T0) and at 4 h (T4), 48 h (T48) and 72 h (T72) after the fifth dose administration (where applicable). The area under the concentration–time curve (AUC) is also reported both graphically and numerically for the 10 participants for whom concentrations were measured.

ranged from 2478 to 12097 for arm B and from 13304 to 50096 for arm C. The decline in viral load at Day 7 and the AEs observed in participants with PK evaluations are listed in Supplementary Table S13. No significant relationship was observed between viral load changes and plasma levels of ivermectin.

4. Discussion

This was the study with the highest dose of ivermectin ever used in a clinical trial for the treatment of COVID-19 or, indeed, for any other condition. The higher dosage (1200 μg/kg for 5 days) showed no safety concerns as no SADRs were observed. The co-primary efficacy outcome was the reduction in viral load on Day 7. The reduction (expressed in $\log_{10}$) was 2.9 for the higher dose (arm C), 2.5 for the lower dose (arm B) and 2.0 for placebo, but the differences were not statistically significant. No significant differences were observed in the clinical outcome either, although the study was not powered sufficiently to detect differences in the secondary outcomes. Concerning AEs, three of the four SAEs (hospitalisation for worsening of the disease) reported were in arm C and 1 in arm B.

Mild or moderate AEs were numerous and reported in all three groups, with the highest proportion in arm C. Of 14 treatment interruptions, 12 were due, precisely, to side effects, of which 11 were in the higher-dose arm (arm C).

The rationale for using a very high dosage was based on the in vitro study published by Caly et al. [8]. The foreseeable plasma concentration after the fifth day of treatment made effective drug levels in pneumocytes plausible, also considering that much higher levels are likely to be achieved in pneumocytes than in plasma [21]. The dosages used in our study were selected according to the PK considerations reported in the study protocol and in the Introduction: the C_{max} after the fifth dose should be of the same order as after a single dose of 2 mg/kg, already found to be safe in healthy volunteers [11]. Our PK results, albeit on a limited number of participants, confirmed our initial hypotheses, indeed showing slightly higher concentrations than foreseen, and reaffirm the biological plausibility of high-dose ivermectin for COVID-19. However, our study failed to show a significant effect on viral load decline.

This study confirms that ivermectin at high dose can be considered safe. However, a high proportion of dropouts, albeit for mild or moderate side effects, was observed in the higher-dose treatment arm. It is true that symptomatic subjects at greater risk of illness progression might be more motivated to continue a treatment in the presence of minor side effects. However, other considerations make a new trial on high-dose ivermectin problematic. First of all, no sign of a possible positive clinical outcome was highlighted. On the contrary, the four participants whose disease worsened enough to cause hospitalisation were all in the treatment arms, and three of these were in the higher-dose arm. Although this is an observation, rather than a statistically valid conclusion, it might be questioned whether ivermectin itself could have at least partly contributed to the clinical worsening. Indeed, there are concerns due to its mechanism of action, which is not directly against the virus but implies inhibition of a host protein involved in intracellular transport [22]. However, the clinical features of the hospitalised participants here were deemed compatible with COVID-19 evolution, and no major neurological signs were observed, as reported previously in cases of serious ivermectin toxicity [23].

Self-administration of veterinary formulations of ivermectin has been widely reported since the initial (and weak) evidence about its possible efficacy against COVID-19. Toxic effects (mostly gastrointestinal distress and neurological symptoms) have been reported following such misuse [24], and the World Health Organization (WHO) has recommended to limit the administration of ivermectin for COVID-19 only for clinical trials [25].

Different groups have carried out systematic reviews with meta-analysis on the use of ivermectin for COVID-19 [26–29]. Results between different works are inconsistent, and the quality of clinical trials included in some reviews has been questioned. On one hand, fraudulent data have been detected; on the other, the sample size of many trials was too small or the study quality was assessed as low [28,30]. Amongst other reviews, a Cochrane meta-analysis has been published [28]. It analysed 14 randomised trials with ivermectin for COVID-19 published until 26 May 2021 including a total of 1678 participants, finding no evidence favouring ivermectin for clinical outcomes or for viral clearance. In all studies, the dosage used was much lower than that of our lower dosage arm, with the exception of the study by Krolewiecki et al. in Argentina [31] that used the same dose as our lower-dose arm (600 μg/kg). The latter study showed a reduction in viral load in treated subjects versus controls in a subgroup of participants in whom the plasma concentration of the drug, measured 4 h after dosing, exceeded 160 ng/mL.

Our study has some limitations. The first is that it failed to reach the planned sample size. However, the conditional power (CP) analysis showed that even reaching the target sample size, the hypothesised effect would hardly be demonstrated (arm B vs. arm A, CP = 0.001; arm C vs. arm A, CP = 0.27). Another limitation was the extreme difficulty in recruiting participants. Approximately 90% of subjects screened were not eligible to be included for various reasons, including a high proportion of refusal to give their consent. Moreover, 79 (84.9%) of the 93 study participants were recruited by the co-ordinating site. Major strengths of this study were the double-blind design, the inclusion of a placebo arm and the lack of any other major deviations from the study protocol.

In conclusion, we did not demonstrate a significant reduction in viral load between ivermectin and placebo, although a trend for the highest dose is apparent. Whether this drug might have clinical efficacy at lower doses remains debated. We believe that our findings further support the WHO recommendation [25] suggesting that it is currently advisable to refrain from administrating ivermectin for the treatment of COVID-19 outside of clinical trials. Considering the reduced tolerability, large, high-dose clinical trials should not be recommended.

Contributors

A complete list of investigators/contributors is reported in the Supplementary material.

Acknowledgments

The authors acknowledge the Data and Safety Monitoring Board (DSMB) members for their enthusiastic and highly professional support: Piero Olliaro, Simone Accordini and Cristiano Chiamulera.

Funding

Insud Pharma & Mundo Sano are thanked for the donation of study medication (ivermectin and placebo), and Alpine Lions Cooperation for the contribution. The study was partly funded by the Italian Ministry of Health 'Fondi Ricerca Corrente' to IRCCS Sacro Cuore Don Calabria Hospital.

Competing interests

None declared.

Ethical approval

The protocol received clearance from the Ethics Committee of INMI–Spallanzani in Rome, which is competent for all COVID-19 trials in Italy [resolution 139/2020 of 28 May 2020].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2021.106516.

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FOI 26-3132 - Document 5

Pathobiology of Aging & Age-related Diseases

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REVIEW ARTICLE

Zinc, aging, and immunosenescence: an overview

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Zinc plays an essential role in many biochemical pathways and participates in several cell functions, including the immune response. This review describes the role of zinc in human health, aging, and immunosenescence. Zinc deficiency is frequent in the elderly and leads to changes similar to those that occur in oxidative inflammatory aging (oxi-inflamm-aging) and immunosenescence. The possible benefits of zinc supplementation to enhance immune function are discussed.

Keywords: *zinc*; *aging*; *immunosenescence*

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Received: 29 July 2014; Revised: 22 October 2014; Accepted: 9 December 2014; Published: 5 February 2015

The importance of micronutrients in nutrition and human health is unquestionable and, among them, zinc (Zn) is an essential trace element whose importance has been especially prominent in the current literature. Its deficiency can play an important role in the aging process and in the etiology of several age-related chronic illnesses such as atherosclerosis, degenerative diseases of the nervous system, immunosenescence, and cancer (1). Zinc is involved in the maintenance of many homeostatic mechanisms, including efficiency of the immune system, acting as a structural and regulatory catalyst ion for the biological activity of many enzymes, proteins, and signal transcription factors, as well as cell proliferation and genome stability (2).

Biological aging is a complex process that, in humans, is associated with changes in all cells, especially in the immune system. Immunosenescence is characterized by a progressive abnormal regulation of immune responses (innate, adaptive) that causes a low-grade inflammatory systemic condition, susceptibility to infections, and a lower efficacy of vaccines (3,4). Currently, much of the physical and biological characteristics of aging are being explained by the unbalance among oxidizing mechanisms (free radicals, oxygen reactive species), antioxidant defenses (5), and inflammatory and antiinflammatory mechanisms. This results in a low-grade, pro-inflammatory state, referred to as inflammatory aging ('inflamm-aging') (6).

The main objective of this review is to approach the recent updates on the role of zinc in human health, the aging process, and immunosenescence. The PubMed, Hinari, Ebsco, Embase, and Dinamed international data bases were searched using the descriptors *zinc, zinc and immunosenescence, zinc and aging, zinc and the immune responses,* and *zinc supplementation.* The searches were conducted between July and December 2013; 968 published articles were found, of which 106 articles that matched the required criteria for the topics included in this review were used. The searches were limited to studies published in the English language, and the priority was articles with systematic review, randomized clinical trials, and clinical reviews.

Homeostasis of zinc

The intracellular homeostasis of Zn (Fig. 1) is regulated by buffer proteins called metallothioneins (MT) which act as storage and transporting proteins of Zn (ZnT and ZIP families) that mediate the sign spotting of intracellular zinc, assigning this element the role of the 'second messenger' (7). The release of ionic Zn is produced through the reduction of thiol groups in the MT molecule. The homeostasis of Zn is altered in the aging process in part as a result of nutritional deficiency common in the elderly, causing them to have decreased levels of this ion (8) and, in addition, high MT levels that cause the sequestration of zinc. This leads to less availability of intracellular ionic Zn (9). Zinc ions are closely linked to various structural and catalytic proteins, enzymes, and transcription factors activated by signal transduction pathways, especially in

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Citation: Pathobiology of Aging & Age-related Diseases 2015, **5**: 25592 - http://dx.doi.org/10.3402/pba.v5.25592

FOI 26-3132 - Document 5

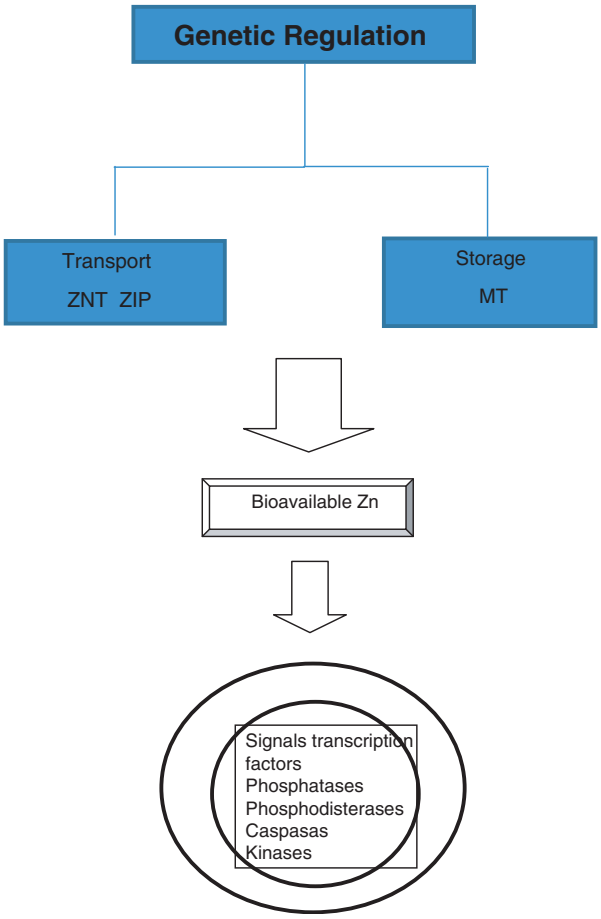


Fig. 1. Homeostasis of zinc.

cells of the immune system. Possible cellular targets of zinc include phosphatases, phosphodiesterases, caspases, kinases, and transcription factors such as nuclear factor kappa B (NF-κB) (10).

Zn activity is genetically regulated as shown by microarray analysis of the total blood ribonucleic acid (RNA) that reveals genes that respond to Zn, particularly those associated with the regulation of the cellular cycle and immune responses. The micronutrients (Zn, Cu, Fe, Mn) play a pivotal role both in maintaining and reinforcing immune and antioxidant performance and in affecting the complex system of genes implicated in encoding necessary proteins for a correct inflammatory immune response (11,12).

Zinc in human health

The importance of zinc for humans was acknowledged in the Middle East (Iran, Egypt), in the early 1960s, in patients with growth retardation, hypogonadism, hepatomegaly, splenomegaly, dry and wrinkled skin, and severe iron deficiency anemia (13). Patients with Zn deficiency had severe immune dysfunction because of which they died from opportunistic infections before the age of 25 (14). In recent years, the data about the effects of Zn deficiency and its supplementation in human health disorders has increased. Zn deficiency causes dysfunction of humoral and cell-mediated immune responses and increases susceptibility to infections. It has also been related to diarrheic diseases with therapeutic benefits being reported in acute diarrhea in children (15,16). A recent review (17) indicates that the Zn supplementation produces reduction in diarrhea and pneumonia mortality in children aged less than 5 in developing countries. There have also been reports of benefits with regard to other illnesses such as enteropathic acrodermatitis, Wilson's disease (in asymptomatic and pre-symptomatic stages), chronic hepatitis C, shigellosis, leprosy, leishmaniasis, and the common cold (18,19).

Current investigations suggest that Zn deficiency increases the risk of neurodegenerative disorders, affecting neurogenesis and increasing neuronal apoptosis, which can cause deficiency in learning and memory. This links Zn deficiency to cerebral aging, depression, Parkinson's disease, and Alzheimer's disease (20–22). It has been postulated that the increased intake of inorganic copper in drinking water can be important in the pathogenesis of Alzheimer's disease. The elevation of the levels of Zn leads to a decrease of copper levels. It has been reported that the treatment with Zn offers protection to patients older than 70 years against the cognitive decline by decreasing the levels of free copper (23). A clinical essay indicates more improvement of symptoms in patients suffering from depression supplemented with Zn and antidepressants than in those who were administered a placebo and antidepressants (24).

Several studies have revealed an association between Zn intake and gastrointestinal cancer. A systematic review of 19 studies up to April 2013 that included 400,000 participants pointed out that a larger intake of zinc led to a reduced risk of colorectal cancer, and a small intake of this microelement was linked to a higher risk of esophageal–gastric cancer in Asia but not in Europe (25). Some authors talk of a possible link between the trace elements copper and zinc (mostly an increase in their association) and the transitional cell carcinoma of the bladder (26,27).

Other studies indicate that Zn can be effective in decreasing the risk of incidence of ocular diseases correlated to aging (28) and the progression of age-related macular degeneration (29). The role of Zn in the distortion of the sense of taste in the elderly is still being discussed (30). In addition, Zn supplementation has proven to be beneficial in decreasing the incidence of infections in the elderly (18) and in sickle cell anemia patients (31).

Abnormal T cell regulation of the inflammatory immune response in aging

The immune system develops significant changes related to age (immunosenescence), which manifest both in innate and adaptive immunity, but they are mainly reflected in

Citation: Pathobiology of Aging & Age-related Diseases 2015, **5**: 25592 - http://dx.doi.org/10.3402/pba.v5.25592

alterations in the bone marrow and in the thymus that cause a change in the composition of the immune cell repertory. This is more significant in T cells (32–34).

Studies performed in healthy octogenarians and nonagenarians have identified an immune risk profile (IRP) of immunosenescence that is distinguished by high numbers of CD8+T cells and low numbers of CD4+T cells (inversion of the index CD4+/CD8+), an increase in the number of dysfunctional and differentiated terminal T cells that were previously exposed to antigens (effectors and memory cells) and the exhaustion of cells that are capable of recognizing and neutralizing new antigens (native or virgins cells) (35–37). Currently, it is being argued whether these changes arise from an intrinsic abnormal regulation of the aging process or from a response to a lifelong continued exposure of the immune system to external stimuli such as infections or other antigenic stimulants (38,39).

In other words, immunosenescence is explained by two hypotheses that are not mutually exclusive: first, the anatomic–physiological decrease of the immune system with aging (intrinsic abnormal regulation) produced, in particular, by the involution of the thymus gland and the decrease of the capacity of auto renovation of the cells of the bone marrow (40,41), and second, the decay that is produced in the immune system due to a lifelong exposure to prolonged or latent infections (42–44) and to a chronic antigenic stimulation (45).

Oxidative inflammatory aging (oxi-inflamm-aging)

Several studies show evidence that a systemic low-grade inflammation characterizes aging with several inflammatory markers, which are significant predictors of mortality in the elderly (46,47). Currently, it is being postulated that immunosenescence is associated with oxidative stress in such a way that the oxidation of proteins alters functionality of immune cells; how this oxidative stress contributes to the chronic inflammatory process is known as oxi-inflamm-aging (48). The oxi-inflamm-aging theory states that oxidative stress associated with aging affects all of the organism's cells, but particularly the cells of regulatory systems (nervous, endocrine, and immune). As a result, there is an inability to preserve redox balance resulting in functional losses that limit the preservation of homeostasis. In the immune system, these changes would be associated with immunosenescence. Activation of improperly regulated transcription factors stimulates the expression of genes that program the production of large quantities of oxidative and inflammatory compounds (cytokines) (49–51).

Other authors believe that inflammatory aging is a consequence and not a cause of immunosenescence. In this sense, it is pointed out that abnormal immune regulation of CD4+ T helper cells in advanced age has been suggested to cause an imbalance of TH1 and TH2 cytokines, with an increase in pro-inflammatory cytokines such as IL-6, IL 1, IL 8, and IL 18, and consequently a chronic systemic inflammation at 'low intensity' (52,53).

Role of zinc in aging and inmunosenescence

Zn is an essential micronutrient required in many cellular processes especially in the normal performance and functioning of the immune system (54). Zn deficiency causes significant decline in the innate and adaptive immune responses and promotes systemic inflammation (55,56). The human adult has a total body content of 2–3 g of Zn, and around 1% of it is replaced daily (57). The recommendations of daily requirements of Zn vary according to different authors and regions. In Spain, 15 mg of elementary Zn for men and 12 mg for women are recommended (58). The daily intake recommended by several reviews for people aged more than 70 are 11 mg for males and 8 mg for women with 40 mg being the top tolerable dosage (56,59). Nine European reports indicate needs of Zn that range from 7 to 14 mg in males and 4.9–9 mg in women (60). Elderly people are a particularly susceptible population to zinc deficiency. Although to this day data are insufficient to determine the frequency of Zn deficiency in the elderly (61), it has been estimated that people older than 65 have an intake of zinc below the 50% recommended level (2). A recent study in a group of 102 elderly European people revealed that 44% of them had Zn deficiency and 20% had high Zn deficiency (62).

Zn deficiency is quite common in elderly, frail people since they often avoid meats and other foods that contain this metal to avoid increasing blood cholesterol levels. In addition, they increase the consumption of refined wheat products deficient in Zn and other fiber-rich foods that contain fitates, which limit the intestinal absorption of this trace element (61,63). Other causes of deficiency of this micronutrient in the elderly include inadequate food chewing, intestinal malabsorption, psychosocial factors such as depression, pharmacologic interactions, and altered subcellular processes (zinc carriers, metallothioneins, divalent metal carrier-1) (2,64). A study performed at nursing homes detected that the lowest serum levels of zinc were found in those patients whose daily life activities were greatly compromised, such as being bed ridden, a low body mass index, and/or increased cognitive decline (65).

Age-related conditions may be related to Zn deficiency by the alteration of intracellular Zn homeostasis, because metallothioneins are unable to release zinc, and transporting proteins (ZIP families) are defective resulting in a low content of intracellular Zn (7). The upregulation of the ZIP genes has some influence in the entrance of Zn into cells, which is more pronounced in lymphocytes from young adults than in lymphocytes from old donors (66). Metallothioneins are antioxidant proteins that release Zn ions for several proteins and enzymes implicated in

FOI 26-3132 - Document 5

antioxidant responses and in the restoration of DNA. In several models of senescent cells, a decreased expression of metallothioneins and intracellular Zn takes place (67).

There is a significant parallel between changes described in immunosenescence and those that are associated with Zn deficiency, among them a reduction of thymus activity and its hormones, a deviation of T helper cells to type TH2 cell activity, a decrease of the response to vaccines, and a deterioration of innate immune cell (phagocytes and NK cells) function (8). The role of Zn and metallothioneins is crucial as they affect NK/NKT development and function. In particular, some of the polymorphisms of metallothioneins are involved in maintaining the innate immune response. The availability of intracellular Zn in aging, many times decreased, compromises this response (68). It has also been demonstrated that Zn deficiency decreases the thymic hormone thymuline, which is necessary for the maturation of T helper cells. This, along with the deviation of the function of TH1 to TH2, causes dysfunction of the immune response mediated by cells (69). Zn is involved in the genetic expression of melatonin recipients and in the proliferation and apoptosis of cells of the thymus and the reactivation of thymuline (70).

Currently, Zn is regarded as a molecule of intracellular signposting, that is, its status becomes altered in response to an extracellular stimulus, acting like a neurotransmitter (71,72). Two types of intracellular signaling of Zn have been identified: an early one, which is induced directly by an extracellular stimulus, and a late one, that depends on changes of expression of the zinc carriers (73,74). In immune cells, the signaling of intracellular Zn modulates its activity (75,76) through the inhibition of NF-κB, a transcription factor that can control many genes of immune response (77,78). In its latent state, NF-κB is sequestered in the cytosol by inhibitory protein IkB, and it is stimulated through the activation of the Ik kinase (IKK) mediated by Zn (79). In cell culture studies, it has been observed that Zn leads protein A 20 that inhibits the enzyme IkB kinase (IKK). This enzyme is capable of inactivating NF-κB, causing a decrease of inflammatory cytokine generation (13,80).

The zinc carrier (ZIP 8) is a transcriptional target of NF-κB and negatively regulates the inflammatory response through modulation of the activity of IKK (80). Recent opinions suggest that during evolution, host defenses and the aging process have been intimately linked with the mechanisms and defensive factors related to the NF-κB system suggesting a common point between aging and immunosenescence (81). As part of new techniques of cell therapy, the induction of metallothioneins provides better protection of the cells because they act as antioxidant, antiinflammatory, and anti-apoptotic agents, increasing the transcriptional regulation of genes implicated in proliferation and cell differentiation (82). Experimental studies in mice, in particular mice with Zn deficiency, have consistently proven that expression of ZIP 8 is remarkably induced in immune innate cells at the beginning of infections with a concomitant increase of signals mediated by NF-κB. This was associated with increased inflammation and an increase in the severity of sepsis (83).

A system of signals has been described in monocytes where a complex interaction occurs among Zn, nitric oxide, cyclic nucleotide signaling, and the IL-1 receptor associated with kinase-1. These act against the production of inflammatory cytokines (84). Also, the function of dendritic cells is affected by disorders in the homeostasis of Zn implicating the transporting proteins (ZIP) during the upregulation induced by lipopolysaccharides and stimulating molecules (85). A study on the effect of Zn ions in the promonocytic leukemia HL-CZ cell line showed that treatment with Zn caused the release of chemokine and inflammatory cytokines through activation of multiple transcription factors related to the immune response (86). Figure 2 illustrates the role of Zn as a signal molecule in the immune system.

Immune cells show a decline in function when there is a decrease in Zn content. Cytotoxicity decreases in monocytes, phagocytosis is reduced in neutrophils, and in B cells apoptosis increases and standard functions of T cells deteriorate, but auto reactivity increases. This has been demonstrated in cell culture studies where the concentrations of Zn vary from 100 to 500 mmol (87). B and T cells with zinc deficiency reveal different patterns of reaction compared to Zn-sufficient cells, consistent with a strong proliferative response followed by stimulation of IL-6 and IL-2 in the first case, with less proliferation followed by stimulation of IL-4 in the second case (88). The closest link among aging, immunosenescence, and Zn deficiency seems to be through the oxi-inflamm-aging process. It has been documented that Zn deficiency increases oxidative stress and causes the generation of inflammatory cytokines, such as IL-1β IL-2, IL-6 and TNF α (1,10,13,14,19,53,61,62,89,90), both *in vitro* and *in vivo*.

Bao et al., using models of sepsis in small animals, noticed that Zn deficiency increased the number of bacteria and the activity of NF-κB in vital organs, including the lungs, with an increase in inflammation, pulmonary damage, and mortality. Zn supplementation right after the initiation of sepsis reversed these effects (91). Wong et al. also found in their studies on aged rats that Zn deficiency, particularly the reduction of intracellular Zn in immune cells, was associated with an increase in inflammation with increasing age (92).

Zinc supplementation in humans

There have been few studies on the effects of Zn supplementation on the human immune response. A clinical trial that included 55 people of both genders (aged between 57 and 87) who were supplemented daily with 45 mg of oral elementary zinc for 12 months reported that the

Citation: Pathobiology of Aging & Age-related Diseases 2015, **5**: 25592 - http://dx.doi.org/10.3402/pba.v5.25592

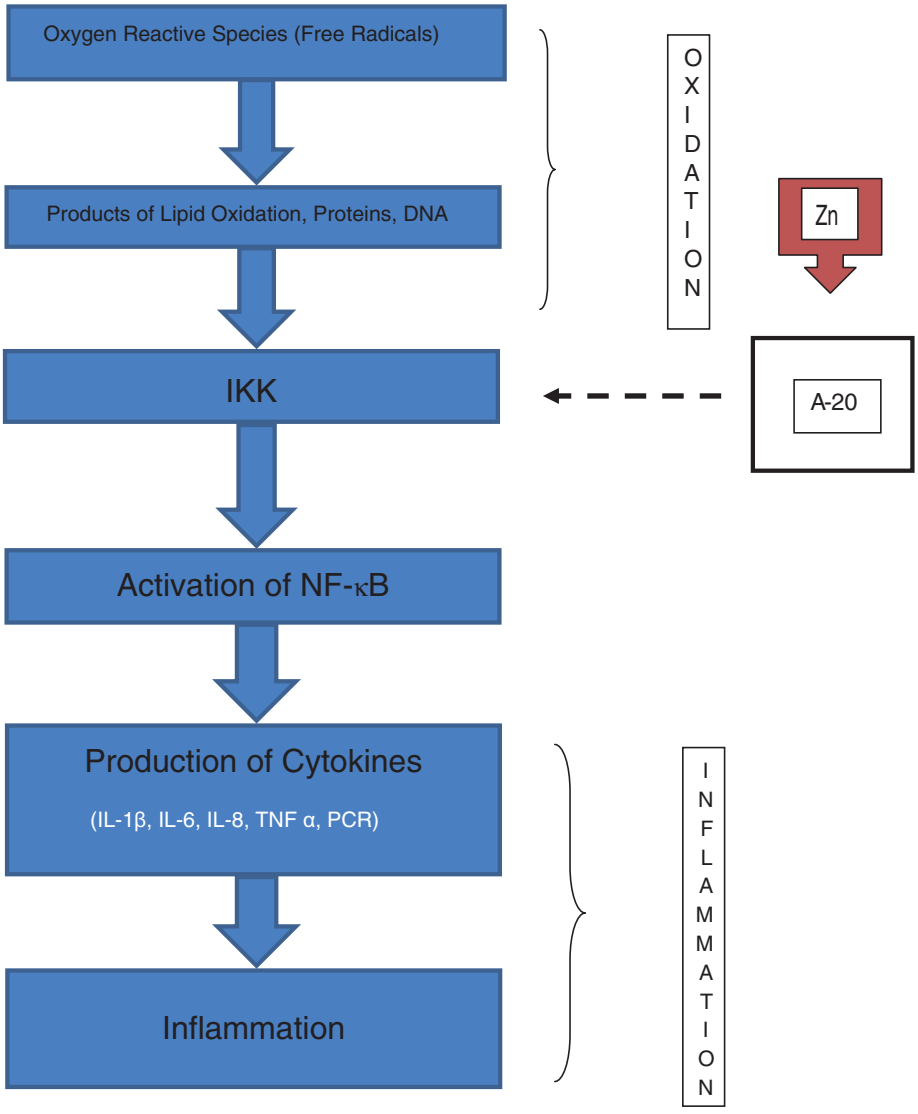


Fig. 2. Zinc, through the activation of protein A-20 inhibits (discontinuous arrow) the IkB kinase (IKK) causing inhibition of the inflammatory cascade. In zinc deficiency, the protein A-20 is 'free', and the activation of NF-κB and the generation of inflammatory cytokines are induced.

incidence of infections and the generation of oxidative stress markers and inflammatory cytokines were remarkably lower in the supplemented group than in the control group (93). Zn deficiency is common in adults suffering from sickle cell anemia. In a study that included 36 sickle cell patients, half received 25 mg of oral Zn once a day for 3 months and the rest a placebo. In the supplemented group, the incidence of infections and the induction of inflammatory cytokines (TNF α, IL-1 β) decreased, while the antioxidant power increased (as shown by a decrease in plasmatic nitrate and nitrite and the products of oxidation of lipids and DNA) (31).

An observational study performed including 420 residents of nursing homes in Boston compared the ones who had low levels of plasmatic Zn (<70 μg/dL) with those with normal levels (≥ 70 μg /dL). In those who had normal concentrations of zinc, the incidence of pneumonia was lower and of decreased duration, with less prescription of new antibiotics, fewer days with the use of antibiotics, and reduction of all causes mortality, compared with the low level group (94). To demonstrate the antiinflammatory and antioxidant effects of Zn, a double-blind randomized trial was performed in 40 people aged 56–83, distributed in a group that received 45 mg of oral Zn gluconate for 6 months and another that received a placebo. In the supplemented group, the inflammatory cytokines (PCR, IL 6) and the markers of oxidative stress (secretory phospholipase A, malondialdehyde, hydroxyalkenals) decreased (95). In a study of long-term hemodialysis patients who had concentrations of serum Zn below the normal (<80 mg/dL), those who were supplemented with Zn for 8 weeks showed remarkably larger percentages of CD4

FOI 26-3132 - Document 5

and CD19 lymphocytes, a higher proportion of CD4/CD8, and a decrease in the proportion Cu/Zn and of the oxidative stress and the inflammatory response (96).

The nutritional state of Zn can influence response to vaccination. A cross-sectional study of 80 elderly people who were inoculated with pneumococcal vaccine revealed that in those who prior to the vaccination had higher levels of Zn, the immune response (IgM) to the vaccine increased by over 10% (97). The supplementation of Zn in children with heart disorders vaccinated against influenza had beneficial effects in decreasing their discomfort (98), which is one of the adverse effects of this vaccine, and also decreased the serum levels of TNF-α. However, in another clinical study in which 140 elder people were vaccinated against hepatitis B, those who were supplemented with Zn sulfate did not show benefits in increasing immunity levels (99).

In an updated systematic review of Zn fortification in diet, only 11 studies were found including 771 participants, mainly children, none on the elderly, with reports of growth rate increase, mostly in those with low weight at birth (100). Efforts have been made to determine the effects of Zn supplements in diarrhea and pneumonia in children where they have demonstrated beneficial effects (18,101).

Other difficulties arise in planning the supplementation of Zn in the elderly, the daily needs vary among populations, the therapeutic dosages are not standardized, and over-administration of Zn can cause toxic effects and side effects on immunity (102). Zn toxicity is relatively unusual. High dietary intake can cause nausea, vomiting, epigastric pain, lethargy, fatigue, and a decline in the immune response, hypocupremia, neutropenia, macrocytic anemia, and sideroblastic anemia (59,103). Currently, it is thought that the evaluation of nutritional Zn is complex due to the fact that the biochemical measurements of this element have limitations in sensitivity and specificity (57). Recently, the measurement of Zn concentrations in fingernails has been used in a unique study in the elderly with and without Alzheimer's disease, but without finding significant differences between these two groups (104). Some authors consider that the selection of elderly people for the supplementation of Zn must be based on low blood concentrations. It is necessary to take into account the genetic background in relation to the polymorphisms of metallothioneins and IL-6. Elderly people who are bearers of the genotype GG (called C negative) in locus IL 6-147G/G develop high levels of IL-6 and metallothioneins, low contents of intracellular Zn, and deterioration of immunity and are the most adequate for Zn supplementation. The bearers of genotypes GC and CC (named C positive) have a satisfactory content of intracellular Zn, and its supplementation can be inappropriate (2,7,105).

Areas of uncertainty for further investigations

Based on what has been investigated thus far, some gaps remain which might be approached in future investigations.

1. A wide field is open relating to nutritional aspects that involve Zn as an essential trace element, and the clinical and biochemical aspects of its deficiency that contribute to a better evaluation and more precise determination of plasma concentrations.
2. The research on the pathogenic mechanisms of Zn deficiency in aging, frailty, immunosenescence, and age-related chronic and degenerative diseases should be continued, in addition to the search for possible therapeutic targets.
3. Clinical trials related to the effects of Zn and its deficiency in the health of the elderly are needed.
4. There is a need for more precise determination of elderly people at risk for Zn deficiency and compounds and ideal dosage for the supplementation of this element.
5. Further studies are needed to determine the efficiency of Zn in fighting infections in the elderly.

Conclusion

Zn is an essential micronutrient for human health in general, and particularly for the elderly, with evidence in cell culture experiments of its involvement as a signaling molecule in the oxidation and inflammatory process that occurs in cell senescence and in immune system dysfunction. Although the body of proof is limited in determining the efficacy of the supplementation of Zn in the prevention and treatment of infections and degenerative processes that accompany old age, promising perspectives are open in the homeostatic regulation of this trace element as a therapeutic objective and an attractive field of anti-aging research. Current evidence suggests that educating the elderly to consume an appropriate diet with foods that contain the necessary Zn requirements would be a health-promoting activity. Clinically, we should not lose sight of the nutritional demands of geriatric patients, taking into account the elements discussed in this review, which possess indisputable practice value.

Conflict of interest and funding

The author has not received any funding or benefits from industry or elsewhere to conduct this study.

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Citation: Pathobiology of Aging & Age-related Diseases 2015, **5**: 25592 - http://dx.doi.org/10.3402/pba.v5.25592

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Citation: Pathobiology of Aging & Age-related Diseases 2015, **5**: 25592 - http://dx.doi.org/10.3402/pba.v5.25592

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PHARMACEUTICAL ONLINE

White Paper

The Advantages And Challenges Of Fixed-Dose Combination Products

Source: Cambrex



The advantages and challenges of fixed-dose combination products: Considerations for design, formulation, manufacturing and analysis

FDC products offer significant advantages for the pharmaceutical industry, including the potential to extend protection of patents and market share, reduce overall manufacturing costs, and add new NDAs to a company's drug development portfolio with shorter and less costly paths to regulatory approval.

The many advantages of fixed-dose combination (FDC) drug products have made them an increasingly attractive option for patients, pharmaceutical companies, physicians, and regulatory agencies. These potential benefits include, but are not limited to, better patient compliance, more convenient dosing associated with improved clinical performance, cost savings for both patients (fewer co-pays) and drug manufacturers (reduced production costs), and extended patent protection and market positioning in the face of competition from generics. These advantages cannot be taken for granted, however. Producing safe and effective FDC products that are also cost-effective and have a high probability of a regulatory approval requires thoughtful product design, access to state-of-the-art formulation and manufacturing technology, and advanced analytical tools.

Introduction

Understanding both the significant competitive advantages that FDC products can offer and the difficulties and potential pitfalls associated with development and manufacturing of FDC drugs in oral solid dosage forms is a critical first step for companies exploring this approach. Recognizing that cost savings, lower risk, and accelerated timelines can be achieved by partnering with a CDMO that has a proven track record of successfully producing FDC products is an important next step. Cambrex has collaborated and partnered with pharmaceutical customers to develop and manufacture FDC drugs that are US FDA approved and are currently in the various stages of clinical development. The experience, know-how, and team of skilled experts that Cambrex brings to these partnerships, combined with its wide range of specialized equipment and technological competencies, ensure the production of high quality FDC drug products that meet their clinical endpoints and receive final approvals from regulatory agencies.

Over the past decade, we have established a vast knowledge base in the area of FDC drugs. Cambrex continues to expand our formulation and manufacturing capabilities through collaborative agreements and by bringing on-stream specialty equipment to enable the production of FDCs. Some examples include hot melt extrusion, wet granulation/ roller compaction, extrusion-spheronization, and fluid bed technology. These manufacturing capabilities provide greater flexibility for our clients in terms of a variety of manufacturing platforms for the production of FDC oral solid dosage forms. These choices include multi-layer tablets, mini-tablets that can be compressed together or combined in capsules, sachets, or stick packs, and microparticulate systems filled into capsules. Cambrex also offers a variety of polymer-coating options to enable controlled release of the different drug substances in an FDC product. Cambrex also has hands-on experience in taste masking that relies on ion exchange technology.

More FDC products flowing into pharma pipelines

The numbers tell the story. Pharmaceutical companies – and the FDA – are embracing FDCs. From 1990 through 2013, the FDA approved 131 FDC products, on average 5.7 per year, the largest number targeting indications related to cardiovascular disease, followed by endocrinology products and treatments for infectious diseases.[1] Of those 131 approved FDCs, 98 were oral dosage forms.

Between 2010 and the end of 2015, of the 655 New Drug Applications (NDAs) approved by the FDA, 63 were for FDCs.[2] This represents nearly 10% of the NDAs approved during that 5-year period and, on average, 12.6 new FDC products per year. As the numbers of FDC products in development and coming to the market increase, the pharmaceutical industry is looking to FDCs as a promising strategy to repurpose, repackage, and expand indications for existing and novel therapeutic agents. The potential to extend the patent life and market exclusivity of a company's APIs is a potent financial incentive.

FDC products can extend patent life and market dominance

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For pharmaceutical companies, FDC products represent an attractive strategy to gain additional patent protection and market exclusivity for a drug approaching patent expiration. "In the US, if an FDC is novel, nonobvious, d the exclusion of competitors from the market can be enforced."[3] Further, "The FDA provides three years of market exclusivity to new [new molecular entity] NME-FDC when the application contains new clinical investigations. If the r ıt and exclusivity life of the FDC will typically be equal to the three year market exclusivity or the of the individual components."[3]

Cambrex et al. compared the effective patent and exclusivity life of FDCs with that of single active ingredients from 1980 through 2012.[3] The FDA approved 28 FDC products containing a NME and 117 FDC products containing only already approved products during the study period. Overall, FDC products without a NME added a median of 9.7 years of patent and market exclusivity protection to that of the single active ingredients. When the company producing the non-NME-FDC was also the sponsor of the single ingredient drugs included in the combination, the non-NME-FDC added a median of 7.73 years; whereas, if those companies differed, the non-NME-FDC added a median of 11.48 years of patent and market exclusivity protection.

Improved convenience and safety

Among the numerous advantages FDCs offer patients, physicians, and pharmaceutical companies, several are particularly noteworthy. One of these is convenience. For patients affected by diseases that require treatment with multiple medications, replacing two or three pills with a single pill can substantially reduce a patient's pill burden. Examples include hypertension (often treated with a combination of beta blockers, ACE inhibitors, and diuretics), diabetes, HIV and other viral infections (typically treated with multi-drug cocktails), cholesterol disorders, and cancer. Pain management is another therapeutic area in which FDCs are gaining increased attention. An emerging strategy combines an opioid drug with an opioid antagonist to counter the "high" brought on by the opioid. This approach achieves effective pain relief, but with a reduced risk of addiction. In addition, FDCs can be used to develop abuse deterrent formulations (ADFs).

FDC products comprise multiple drug substances that are taken in a single dose. The components of an FDC cannot be changed or separated. A physician can prescribe more or less of the combination product, but the ratio of the individual drug components cannot be altered. As a result, FDC products may help prevent medication errors and enable better control of dosing. Dosing is not left up to physicians; it is built into the FDC product and based on clinical trial results.

The goal of combination drug treatment is not only the delivery of two or more therapeutic agents that have different mechanisms of action. FDC products can also take advantage of potential synergistic effects between the individual drug substances, which could improve overall effectiveness and perhaps allow for simplified and reduced dosing, which may lower the risk of adverse effects.

Cost savings

A decreased pill burden is only one example of how FDCs are more convenient for patients. Replacing two or three pills with a single FDC drug product also translates into less time spent filling (and refilling) prescriptions. With an FDC product, physician offices also have fewer prescriptions and renewals to process. Taking this a step further, fewer prescriptions means fewer co-pays for patients, saving them money. Theoretically, at least, pharmaceutical companies should also be able to save money producing a single-dose FDC product, compared to what it would cost to manufacture, package, and market two or three individual drugs.

Accelerated regulatory pathway

Furthermore, compared to the cost of developing a new API, substantial savings can be realized when developing an FDC comprised of previously approved new chemical entities or their generic counterparts. The FDA allows for an abbreviated clinical development program that can speed the path to approval if sufficient drug safety and tolerability data and supporting efficacy data are already available. Companies can save valuable time and resources as well by taking advantage of the FDA's 505(b)(2) approval pathway.

Over the past several years, many approved FDC products have used the 505(b)(2) pathway. In more than half of those cases, the companies still had to perform at least some Phase II and III clinical studies.[4] Specifically, about two thirds of the FDCs approved using the 505(b)(2) route have required at least one Phase II or Phase III study, and approximately one-third needed more than four Phase II/III studies.[4]

> Examples of FDA-approved oral solid dosage form FDC products that greatly reduce the pill burden for patients with HIV infection are:
>
> - Gilead's Genvoya (elvitegravir 150mg/obicistat 150mg/emtricitabine 200mg/tenofovir alafenamide 10mg)
> - Viiv Healthcare's Triumeq (abacavir 600mg/ dolutegravir 50mg/lamivudine 300mg)

An updated European Medicines Agency (EMA) guideline on the clinical development of FDC drugs is expected to be issued during the fourth quarter of 2017. It details the evidence that companies need to present to support authorization of FDCs that contain two or more already approved or new active substances, whether small molecule drugs or biologics.[5] Initially adopted in March 2017, the newly revised EMA guidelines require data to "support the rationale for combined use of the active substances." Simply providing previous evidence of combined use "will not suffice to establish the positive benefit/risk of the combination."

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The EMA states: "Specific considerations apply for fixed combination medicinal products where the active substances have different – but related – therapeutic indications and different pharmacological targets, e.g. a fixed combinatic patients at high cardiovascular risk containing a lipid-modifying agent and an antihypertensive agent. A relevant contribution of all active substances and existence of a positive benefit/risk for these fixed combination medicinal Furthermore, "as a minimum requirement, in the absence of clinical trial data studying the spec combination on clinical outcome, the potential for PK and PD interactions should be establishec individual active substances may be modified by their combination. Usually, PK data (a DDI study) will suffice."

Developing and manufacturing FDCs factors to consider

At the outset, when designing an FDC drug, API selection is the foundation on which creating a successful product rests. Selecting the APIs to include in a two-drug or three-drug FDC product should focus on maximizing the benefit-to-risk ratio by identifying combinations that have the potential to improve efficacy while avoiding any new safety or tolerability issues. Key drivers for pharmaceutical companies include the ability to repurpose already approved therapeutic agents with a low risk of toxicity, to combine and repackage drugs commonly prescribed together, and to pair their APIs with popular off-patent drugs or generic compounds.

Important factors to consider in designing an FDC product include the following:

- Differing pharmacokinetic and target release profiles of the component drug substances
- Physical and chemical compatibility of the APIs
- The target patient population (age, severity of illness and/or disability)
- The full range of oral solid dosage form options
- Total development and production costs, including formulation, manufacturing, and packaging

Collaboration with ExxPharma Therapeutics

Hot melt extrusion is a technique used to enhance the solubility of drug substances. Cambrex entered into a collaboration with ExxPharma Therapeutics LCC, a pioneer in HME, to apply the technique in the development and manufacturing of drug products. The aim of HME is to minimize exposure of a drug substance to elevated temperatures during manufacturing – temperatures above a drug's melting point that could lead to degradation. HME may offer the advantage of higher drug loading versus a spray-dried dispersion containing the same API. This is particularly useful in an FDC where higher drug loading may be necessary. HME also helps ensure the stability of a drug substance by eliminating air during melt extrusion, thereby limiting oxidative degradation.

HME technology offers additional advantages as well as:[6]

- *Small footprint of the extruder*
- *Process uses no organic solvents, is continuous, and is easily scalable*
- *HME does not require an extra densification step*
- *Unlike with spray-dried solid dispersion technology, the material generated can be blended directly with other FDC components and compressed into tablets*

The use of HME to produce FDC drug products simplifies technology transfer and scale-up, accelerating the time to market, and contributes to improved overall product quality.

Working with a CDMO partner

Theoretically, at least, there should be a cost advantage in the production of a single FDC drug compared to the costs of manufacturing, analyzing, testing, packaging, and marketing two or three individual products. However, the need for specialized equipment and advanced methods and technologies across the formulation, analytical, and manufacturing workflows in order to produce these more complex combination dosage forms can increase capital equipment costs. Outsourcing FDC product formulation and manufacturing, and partnering with an experienced CDMO that has the infrastructure, capital equipment (and competency to run said equipment), technology, and skilled personnel can reduce the costs – both capital (capex) and operating (opex) expenses – and the risks associated with creating high quality FDC products.

The main challenges in FDC product development, which a knowledgeable CDMO partner can help overcome, include the following:

- Designing a stable formulation
- Optimizing the bioavailability and delivery of the individual drug substances
- Understanding the potential for – and avoiding – unwanted interactions and interferences between the API components
- Acquiring the necessary capital equipment – investment of millions of dollars in new equipment required for production of an FDC product

Access to Cambrex's wide range of formulation and manufacturing technologies allows our sponsor partners to identify the most appropriate, efficient, and cost-effective development approach for any particular FDC product. Cambrex can apply all of its technologies and approaches from development scale (500g) through to full-scale commercial manufacturing. This high degree of

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flexibility, combined with ease of access to the equipment and technological resources all in one location, not only helps ensure a productive and highly collaborative partnership, but also allows Cambrex to offer aggressive timel

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Cambrex's specialized equipment and techniques include bilayer presses, wet granulation/rolle solvent pellet layering, and extrusion-spheronization. Through a partnership with ExxPharma T lt extrusion (see "Collaboration with ExxPharma Therapeutics") to improve manufacturing of dru ıtive formulation development and cutting-edge manufacturing technology, Cambrex can create solid dosage forms that include multi-layer tablets, tablet-in-tablet configurations, mini-tablets, and microparticulate systems. Mini-tablets can be combined in capsules, or packaged into sachets or stick packs for resuspension and use in pediatric delivery systems. Similarly, microparticulate systems of individual APIs can be combined in various dosage forms and strengths for adult or pediatric dosing.

Cambrex can use a range of polymer coating techniques to segregate FDC product components that may be incompatible and to provide sustained- or delayed release control of component APIs. Traditional sugar and polymer-coating methods and Cambrex's novel ion exchange technique offer a range of effective options for taste masking.

Cambrex has in place a full range of analytical capabilities to develop and validate stability-indicating methods for final release of an FDC product. One commonly used technique is ultra-high pressure liquid chromatography (UPLC), which is very effective in separating actives and their respective related substances. The ability to use a single analytical method to obtain the necessary data reduces the time and cost associated with generating final or stability quality control (QC) results.

Finally, from a regulatory perspective, Cambrex's strong record of collaborating with pharmaceutical companies to bring FDC products to the market includes the knowledge and experience in gathering and preparing the data and accompanying analysis and reports needed for regulatory filing.

Conclusions

A recent case study describing specific projects that Cambrex completed for two large pharmaceutical customers demonstrates the broad-ranging technological capabilities and expertise Cambrex can offer.7 In one collaboration, Cambrex developed and manufactured a complex three-API FDC product in which one of the APIs was incompatible with the other 2. In the second project, the challenge involved combining 2 APIs with different release profiles. These examples, and the creative solutions Cambrex was able to deliver using multi-layer tablets, mini-tablets, and polymer-coating technology, exemplify the novel types of FDC products on the market and in development that offer important advantages for patients, physicians, and the pharmaceutical industry.

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FOI 26-3132 - Document 5

Antiviral Research 178 (2020) 104787

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 *in vitro*

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A B S T R A C T

Although several clinical trials are now underway to test possible therapies, the worldwide response to the COVID-19 outbreak has been largely limited to monitoring/containment. We report here that Ivermectin, an FDA-approved anti-parasitic previously shown to have broad-spectrum anti-viral activity *in vitro*, is an inhibitor of the causative virus (SARS-CoV-2), with a single addition to Vero-hSLAM cells 2 h post infection with SARS-CoV-2 able to effect ~5000-fold reduction in viral RNA at 48 h. Ivermectin therefore warrants further investigation for possible benefits in humans.

1. Introduction

Ivermectin is an FDA-approved broad spectrum anti-parasitic agent (Gonzalez Canga et al., 2008) that in recent years we, along with other groups, have shown to have anti-viral activity against a broad range of viruses (Gotz et al., 2016; Lundberg et al., 2013; Tay et al., 2013; Wagstaff et al., 2012) *in vitro*. Originally identified as an inhibitor of interaction between the human immunodeficiency virus-1 (HIV-1) integrase protein (IN) and the importin (IMP) α/β1 heterodimer responsible for IN nuclear import (Wagstaff et al., 2011), Ivermectin has since been confirmed to inhibit IN nuclear import and HIV-1 replication (Wagstaff et al., 2012). Other actions of ivermectin have been reported (Mastrangelo et al., 2012), but ivermectin has been shown to inhibit nuclear import of host (eg. (Kosyna et al., 2015; van der Watt et al., 2016)) and viral proteins, including simian virus SV40 large tumour antigen (T-ag) and dengue virus (DENV) non-structural protein 5 (Wagstaff et al., 2012, Wagstaff et al., 2011). Importantly, it has been demonstrated to limit infection by RNA viruses such as DENV 1-4 (Tay et al., 2013), West Nile Virus (Yang et al., 2020), Venezuelan equine encephalitis virus (VEEV) (Lundberg et al., 2013) and influenza (Gotz et al., 2016), with this broad spectrum activity believed to be due to the reliance by many different RNA viruses on IMPα/β1 during infection (Caly et al., 2012; Jans et al., 2019). Ivermectin has similarly been shown to be effective against the DNA virus pseudorabies virus (PRV) both *in vitro* and *in vivo*, with ivermectin treatment shown to increase survival in PRV-infected mice (Lv et al., 2018). Efficacy was not observed for ivermectin against Zika virus (ZIKV) in mice, but the authors acknowledged that study limitations justified re-evaluation of ivermectin's anti-ZIKV activity (Ketkar et al., 2019). Finally, ivermectin was the focus of a phase III clinical trial in Thailand in 2014–2017, against DENV infection, in which a single daily oral dose was observed to be safe and resulted in a significant reduction in serum levels of viral NS1 protein, but no change in viremia or clinical benefit was observed (see below) (Yamasmith et al., 2018).

The causative agent of the current COVID-19 pandemic, SARS-CoV-2, is a single stranded positive sense RNA virus that is closely related to severe acute respiratory syndrome coronavirus (SARS-CoV). Studies on SARS-CoV proteins have revealed a potential role for IMPα/β1 during infection in signal-dependent nucleocytoplasmic shutting of the SARS-CoV Nucleocapsid protein (Rowland et al., 2005; Timani et al., 2005; Wulan et al., 2015), that may impact host cell division (Hiscox et al., 2001; Wurm et al., 2001). In addition, the SARS-CoV accessory protein ORF6 has been shown to antagonize the antiviral activity of the STAT1 transcription factor by sequestering IMPα/β1 on the rough ER/Golgi membrane (Frieman et al., 2007). Taken together, these reports suggested that ivermectin's nuclear transport inhibitory activity may be effective against SARS-CoV-2.

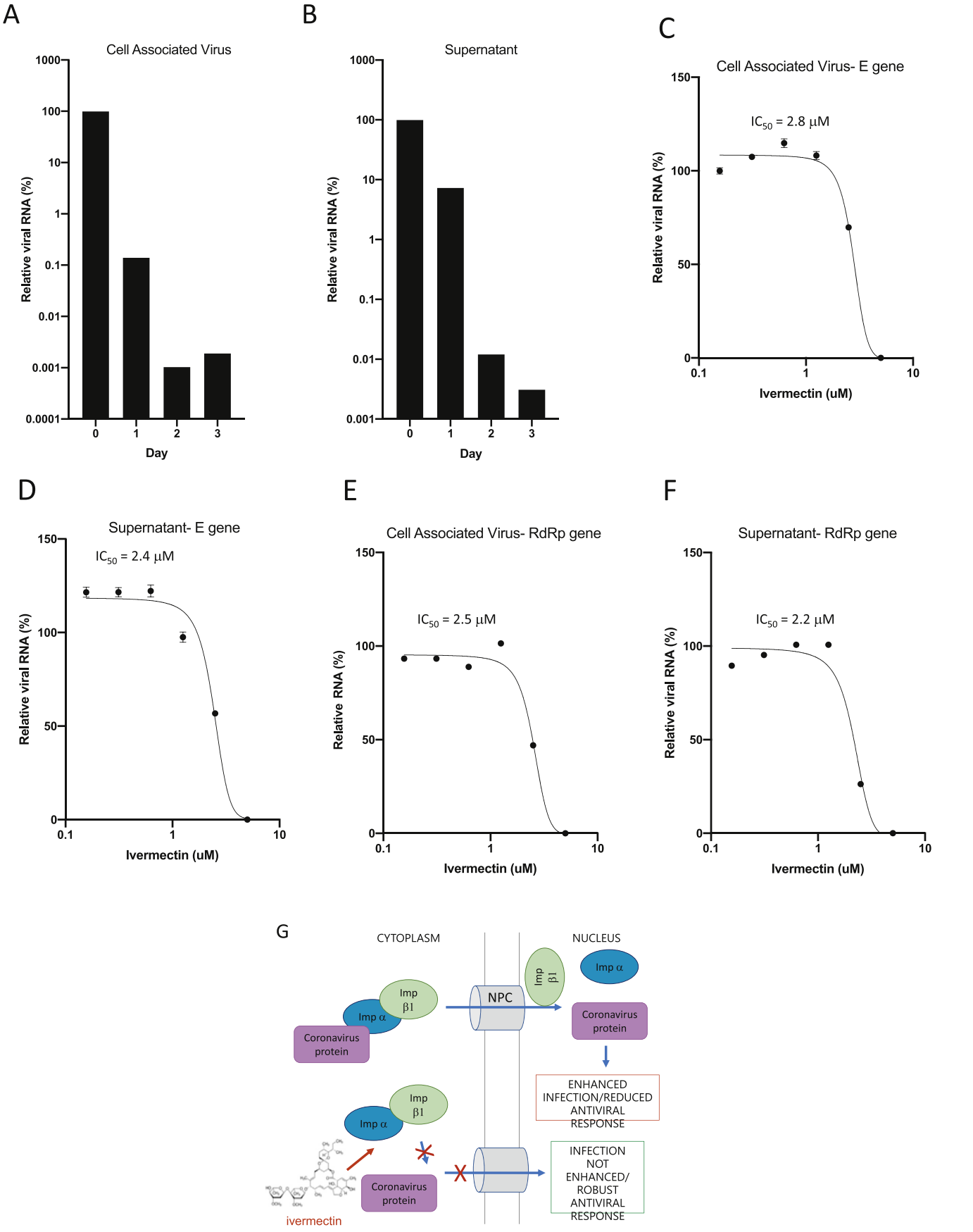
To test the antiviral activity of ivermectin towards SARS-CoV-2, we infected Vero/hSLAM cells with SARS-CoV-2 isolate Australia/VIC01/2020 at an MOI of 0.1 for 2 h, followed by the addition of 5 μM ivermectin. Supernatant and cell pellets were harvested at days 0–3 and analysed by RT-PCR for the replication of SARS-CoV-2 RNA (Fig. 1A/B).

The authors would like readers to be aware of the following letter issued by the FDA titled: "Do Not Use Ivermectin Intended for Animals as Treatment for COVID-19 in Humans" at https://www.fda.gov/animal-veterinary/product-safety-information/fda-letter-stakeholders-do-not-use-ivermectin-intended-animals-treatment-covid-19-humans.

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https://doi.org/10.1016/j.antiviral.2020.104787
Received 18 March 2020; Received in revised form 27 March 2020; Accepted 29 March 2020
Available online 03 April 2020
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FOI 26-3132 - Document 5
Antiviral Research 178 (2020) 104787



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Fig. 1. Ivermectin is a potent inhibitor of the SARS-CoV-2 clinical isolate Australia/VIC01/2020. Vero/hSLAM cells were in infected with SARS-CoV-2 clinical isolate Australia/VIC01/2020 (MOI = 0.1) for 2 h prior to addition of vehicle (DMSO) or Ivermectin at the indicated concentrations. Samples were taken at 0–3 days post infection for quantitation of viral load using real-time PCR of cell associated virus (**A**) or supernatant (**B**). IC_{50} values were determined in subsequent experiments at 48 h post infection using the indicated concentrations of Ivermectin (treated at 2 h post infection as per **A/B**). Triplicate real-time PCR analysis was performed on cell associated virus (**C/E**) or supernatant (**D/F**) using probes against either the SARS-CoV-2 E (**C/D**) or RdRp (**E/F**) genes. Results represent mean ± SD (n = 3). 3 parameter dose response curves were fitted using GraphPad prism to determine IC_{50} values (indicated). **G.** Schematic of ivermectin's proposed antiviral action on coronavirus. IMPα/β1 binds to the coronavirus cargo protein in the cytoplasm (top) and translocates it through the nuclear pore complex (NPC) into the nucleus where the complex falls apart and the viral cargo can reduce the host cell's antiviral response, leading to enhanced infection. Ivermectin binds to and destabilises the Impα/β1 heterodimer thereby preventing Impα/β1 from binding to the viral protein (bottom) and preventing it from entering the nucleus. This likely results in reduced inhibition of the antiviral responses, leading to a normal, more efficient antiviral response.

At 24 h, there was a 93% reduction in viral RNA present in the supernatant (indicative of released virions) of samples treated with ivermectin compared to the vehicle DMSO. Similarly a 99.8% reduction in cell-associated viral RNA (indicative of unreleased and unpackaged virions) was observed with ivermectin treatment. By 48 h this effect increased to an ~5000-fold reduction of viral RNA in ivermectin-treated compared to control samples, indicating that ivermectin treatment resulted in the effective loss of essentially all viral material by 48 h. Consistent with this idea, no further reduction in viral RNA was observed at 72 h. As we have observed previously (Lundberg et al., 2013; Tay et al., 2013; Wagstaff et al., 2012), no toxicity of ivermectin was observed at any of the timepoints tested, in either the sample wells or in parallel tested drug alone samples.

To further determine the effectiveness of ivemectin, cells infected with SARS-CoV-2 were treated with serial dilutions of ivermectin 2 h post infection and supernatant and cell pellets collected for real-time RT-PCR at 48 h (Fig. 1C/D). As above, a > 5000 reduction in viral RNA was observed in both supernatant and cell pellets from samples treated with 5 μM ivermectin at 48 h, equating to a 99.98% reduction in viral RNA in these samples. Again, no toxicity was observed with ivermectin at any of the concentrations tested. The IC50 of ivermectin treatment was determined to be ~2 μM under these conditions. Underlining the fact that the assay indeed specifically detected SARS-CoV-2, RT-PCR experiments were repeated using primers specific for the viral RdRp gene (Fig. 1E/F) rather than the E gene (above), with nearly identical results observed for both released (supernatant) and cell-associated virus.

Taken together these results demonstrate that ivermectin has antiviral action against the SARS-CoV-2 clinical isolate *in vitro*, with a single dose able to control viral replication within 24–48 h in our system. We hypothesise that this is likely through inhibiting IMPα/β1-mediated nuclear import of viral proteins (Fig. 1G), as shown for other RNA viruses (Tay et al., 2013; Wagstaff et al., 2012; Yang et al., 2020); confirmation of this mechanism in the case of SARS-CoV-2, and identification of the specific SARS-CoV-2 and/or host component(s) impacted (see (Yang et al., 2020)) is an important focus future work in this laboratory. Ultimately, development of an effective anti-viral for SARS-CoV-2, if given to patients early in infection, could help to limit the viral load, prevent severe disease progression and limit person-person transmission. Benchmarking testing of ivermectin against other potential antivirals for SARS-CoV-2 with alternative mechanisms of action (Dong et al., 2020; Elfiky, 2020; Gordon et al., 2020; Li and De Clercq, 2020; Wang et al., 2020) would thus be important as soon as practicable. This Brief Report raises the possibility that ivermectin could be a useful antiviral to limit SARS-CoV-2, in similar fashion to those already reported (Dong et al., 2020; Elfiky, 2020; Gordon et al., 2020; Li and De Clercq, 2020; Wang et al., 2020); until one of these is proven to be beneficial in a clinical setting, all should be pursued as rapidly as possible.

Ivermectin has an established safety profile for human use (Gonzalez Canga et al., 2008; Jans et al., 2019; Buonfrate et al., 2019), and is FDA-approved for a number of parasitic infections (Gonzalez Canga et al., 2008; Buonfrate et al., 2019). Importantly, recent reviews and meta-analysis indicate that high dose ivermectin has comparable safety as the standard low-dose treatment, although there is not enough evidence to make conclusions about the safety profile in pregnancy (Navarro et al., 2020; Nicolas et al., 2020). The critical next step in further evaluation for possible benefit in COVID-19 patients will be to examine a multiple addition dosing regimen that mimics the current approved usage of ivermectin in humans. As noted, ivermectin was the focus of a recent phase III clinical trial in dengue patients in Thailand, in which a single daily dose was found to be safe but did not produce any clinical benefit. However, the investigators noted that an improved dosing regimen might be developed, based on pharmacokinetic data (Yamasmith et al., 2018). Although DENV is clearly very different to SARS-CoV-2, this trial design should inform future work going forward. Altogether the current report, combined with a known-safety profile, demonstrates that ivermectin is worthy of further consideration as a possible SARS-CoV-2 antiviral.

2. Methods

2.1. Cell culture, viral infection and drug treatment

Vero/hSLAM cells (Ono et al., 2001) were maintained in Earle's Minimum Essential Medium (EMEM) containing 7% Fetal Bovine Serum (FBS) (Bovogen Biologicals, Keilor East, AUS) 2 mM L-Glutamine, 1 mM Sodium pyruvate, 1500 mg/L sodium bicarbonate, 15 mM HEPES and 0.4 mg/ml geneticin at 37 °C, 5% CO_2. Cells were seeded into 12-well tissue culture plates 24 h prior to infection with SARS-CoV-2 (Australia/VIC01/2020 isolate) at an MOI of 0.1 in infection media (as per maintenance media but containing only 2% FBS) for 2 h. Media containing inoculum was removed and replaced with 1 mL fresh media (2% FBS) containing Ivermectin at the indicated concentrations or DMSO alone and incubated as indicated for 0–3 days. At the appropriate timepoint, cell supernatant was collected and spun for 10 min at 6,000 g to remove debris and the supernatant transferred to fresh collection tubes. The cell monolayers were collected by scraping and resuspension into 1 mL fresh media (2% FBS). Toxicity controls were set up in parallel in every experiment on uninfected cells.

2.2. Generation of SARS-CoV-2 cDNA

RNA was extracted from 200 μL aliquots of sample supernatant or cell suspension using the QIAamp 96 Virus QIAcube HT Kit (Qiagen, Hilden, Germany) and eluted in 60 μl. Reverse transcription was performed using the BioLine SensiFAST cDNA kit (Bioline, London, United Kingdom), total reaction mixture (20 μl), containing 10 μL of RNA extract, 4 μl of 5x TransAmp buffer, 1 μl of Reverse Transcriptase and 5 μl of Nuclease free water. The reactions were incubated at 25 °C for 10 min, 42 °C for 15 min and 85 °C for 5 min.

2.3. Detection of SARS-CoV-2 using a TaqMan Real-time RT-PCR assay

TaqMan RT-PCR assay were performed using 2.5 μl cDNA, 10 μl Primer Design PrecisonPLUS qPCR Master Mix 1 μM Forward (5′- AAA TTC TAT GGT GGT TGG CAC AAC ATG TT-3′), 1 μM Reverse (5′- TAG GCA TAG CTC TRT CAC AYT T-3′) primers and 0.2 μM probe (5′-FAM-TGG GTT GGG ATT ATC-MGBNFQ-3′) targeting the BetaCoV RdRp (RNA-dependent RNA polymerase) gene or Forward (5′-ACA GGT ACG

TTA ATA GTT AAT AGC GT -3′), 1 μM Reverse (5′-ATA TTG CAG CAG TAC GCA CAC A-3′) primers and 0.2 μM probe (5′-FAM-ACA CTA GCC ATC CTT ACT GCG CTT CG-286 NFQ-3′) targeting the BetaCoV E-gene (Corman et al., 2020). Real-time RT-PCR assays were performed on an Applied Biosystems ABI 7500 Fast real-time PCR machine (Applied Biosystems, Foster City, CA, USA) using cycling conditions of 95 °C for 2 min, 95 °C for 5 s, 60 °C for 24 s. SARS-CoV-2 cDNA (Ct~28) was used as a positive control. Calculated Ct values were converted to fold-reduction of treated samples compared to control using the ΔCt method (fold changed in viral RNA = 2^ΔCt) and expressed as % of DMSO alone sample. IC50 values were fitted using 3 parameter dose response curves in GraphPad prism.

Funding

This work was supported by a National Breast Cancer Foundation Fellowship, Australia (ECF-17-007) for KMW and an National Health and Medical Research Council (NHMRC), Australia Senior Prinicple Research Fellow (SPRF) (APP1103050) for DAJ.

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FOI 26-3132 - Document 5

JOURNAL OF MEDICAL MICROBIOLOGY

SHORT COMMUNICATION

Carlucci *et al.*, *Journal of Medical Microbiology* 2020;69:1228–1234

DOI 10.1099/jmm.0.001250

MICROBIOLOGY SOCIETY

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Zinc sulfate in combination with a zinc ionophore may improve outcomes in hospitalized COVID-19 patients

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Abstract

Introduction. COVID-19 has rapidly emerged as a pandemic infection that has caused significant mortality and economic losses. Potential therapies and prophylaxis against COVID-19 are urgently needed to combat this novel infection. As a result of *in vitro* evidence suggesting zinc sulphate may be efficacious against COVID-19, our hospitals began using zinc sulphate as add-on therapy to hydroxychloroquine and azithromycin.

Aim. To compare outcomes among hospitalized COVID-19 patients ordered to receive hydroxychloroquine and azithromycin plus zinc sulphate versus hydroxychloroquine and azithromycin alone.

Methodology. This was a retrospective observational study. Data was collected from medical records for all patients with admission dates ranging from 2 March 2020 through to 11 April 2020. Initial clinical characteristics on presentation, medications given during the hospitalization, and hospital outcomes were recorded. The study included patients admitted to any of four acute care NYU Langone Health Hospitals in New York City. Patients included were admitted to the hospital with at least one positive COVID-19 test and had completed their hospitalization. Patients were excluded from the study if they were never admitted to the hospital or if there was an order for other investigational therapies for COVID-19.

Results. Patients taking zinc sulphate in addition to hydroxychloroquine and azithromycin (n=411) and patients taking hydroxychloroquine and azithromycin alone (n=521) did not differ in age, race, sex, tobacco use or relevant comorbidities. The addition of zinc sulphate did not impact the length of hospitalization, duration of ventilation or intensive care unit (ICU) duration. In univariate analyses, zinc sulphate increased the frequency of patients being discharged home, and decreased the need for ventilation, admission to the ICU and mortality or transfer to hospice for patients who were never admitted to the ICU. After adjusting for the time at which zinc sulphate was added to our protocol, an increased frequency of being discharged home (OR 1.53, 95% CI 1.12–2.09) and reduction in mortality or transfer to hospice among patients who did not require ICU level of care remained significant (OR 0.449, 95% CI 0.271–0.744).

Conclusion. This study provides the first *in vivo* evidence that zinc sulphate may play a role in therapeutic management for COVID-19.

INTRODUCTION

The World Health Organization has declared a pandemic due to the spread of the coronavirus disease of 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) [1, 2]. Despite limited and conflicting data, the U.S. Food and Drug Administration authorized the emergency use of hydroxychloroquine for the treatment of COVID-19 with or without azithromycin. Hydroxychloroquine was thought to be efficacious partly based on *in vitro* activity against SARS-CoV-2 [3]. However, clinical data in humans has yielded mixed and disappointing results [4–7]. In spite of this, hydroxychloroquine may still have a role at blocking coronavirus replication, when used in combination with zinc, which inhibits coronavirus RNA polymerase activity.

Zinc is an essential trace element that is required for the maintenance of adaptive and innate immune responses [8].

Received 13 May 2020; Accepted 12 August 2020; Published 15 September 2020

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Keywords: covid 19; coronavirus; hydroxychloroquine; ionophore; mortality; zinc.

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 FOI 26-3132 - Document 5

The benefits of zinc have previously been recognized for its therapeutic use against other respiratory viruses including those that cause the common cold [9–11]. Zinc has also been observed to improve pneumonia in children and its deficiency is associated with pneumonia in the elderly [9, 12, 13]. Implicating a role for zinc in COVID-19, zinc inhibits viral RNA-dependent RNA polymerase, and has been shown to do this *in vitro* against SARS-CoV [14]. When combined with a zinc ionophore, such as hydroxychloroquine, cellular uptake is increased making it more likely to achieve suitably elevated intracellular zinc concentrations for viral inhibition [10, 15]. This combination is already being tested as a prophylactic regimen in a prospectively followed cohort (NCT04326725) and in a randomized clinical trial (NCT04377646). Other trials are also investigating this regimen for therapeutic efficacy (NCT04370782, NCT04373733).

As New York became the epicentre of the pandemic, hospitals in the area quickly adopted investigational therapies, including the use of hydroxychloroquine and azithromycin. Given this proposed synergistic effect of zinc with hydroxychloroquine, practices at NYULH changed and the addition of zinc sulphate 220 mg PO BID along with hydroxychloroquine 400 mg once followed by 200 mg PO BID with azithromycin 500 mg once daily became part of the treatment approach for patients admitted to the hospital with COVID-19. This study sought to investigate outcomes among patients who received hydroxychloroquine and azithromycin alone compared to those who received triple therapy of hydroxychloroquine and azithromycin plus zinc sulphate.

METHODS

We performed a retrospective analysis of data from patients hospitalized with confirmed SARS-CoV-2 infection at NYU Langone Health. Data was collected from electronic medical records (Epic Systems, Verona, WI, USA) for all patients being treated with admission dates ranging from 2 March 2020 through 11 April 2020. Patients were admitted to any of four acute care NYU Langone Health hospitals across New York City. COVID-19 positivity was determined by real-time reverse-transcriptase PCR (RT-PCR) of nasopharyngeal or oropharyngeal swabs.

Patients were included in the study if they were admitted to the hospital, had at least one positive test for COVID-19, were ordered to receive hydroxychloroquine and azithromycin, and had either been discharged from the hospital, transitioned to hospice or expired. Patients were excluded from the study if they were never admitted to the hospital or if there was an order for other investigational therapies for COVID-19, including tocilizumab, nitazoxanide, rituximab, anakinra, remdesivir or lopinavir/ritonavir during the course of their hospitalization to avoid potential confounding effects of these medications. We collected demographics as reported by the patient and any past medical history of hypertension, hyperlipidemia, coronary artery disease, heart failure, chronic obstructive pulmonary disease, asthma, malignancy other than non-melanoma skin malignancy and diabetes. We also recorded vital signs on admission, the first set of laboratory results as continuous variables, and relevant medications as categorical variables, including NSAIDs, anticoagulants, antihypertensive medications and corticosteroids ordered at any point during the course of the hospitalization. We compared clinical outcomes of patients who received a combination of zinc sulphate plus hydroxychloroquine, a zinc ionophore, to those that received hydroxychloroquine without zinc supplementation.

Statistics

Patients were categorized based on their exposure to hydroxychloroquine (400 mg load followed by 200 mg twice daily for 5 days) and azithromycin (500 mg once daily) alone or with zinc sulphate (220 mg capsule containing 50 mg elemental zinc twice daily for 5 days) as treatment in addition to standard supportive care. Descriptive statistics are presented as mean and standard deviation or mean and interquartile range for continuous variables and frequencies for categorical variables. Normality of distribution for continuous variables was assessed by measures of skewness and kurtosis, deeming the dataset appropriate for parametric or nonparametric analysis. A two-tailed Student's *t*-test was used for parametric analysis, and a Mann–Whitney U test was used for nonparametric data analysis. Pearson's chi-squared test was used to compare categorical characteristics between the two groups of patients. Linear regression for continuous variables or logistic regression for categorical variables was performed with the presence of zinc as the predictor variable and outcome measures [duration of hospital stay, duration of mechanical ventilation, maximum oxygen flow rate, average oxygen flow rate, average FiO2, maximum FiO2, admission to the intensive care unit (ICU), duration of ICU stay, death/hospice, need for intubation, and discharge destination], as dependent variables. Data was log transformed where appropriate to render the distribution normal for linear regression analysis. Multivariate logistic regression was used to adjust for the timing that our protocol changed to include zinc therapy using admission before or after 25 March as a categorical variable. *P* values less than 0.05 were considered to be significant. All analyses were performed using STATA/SE 16.0 software (STATA Corp.).

Study approval

The study was approved by the NYU Grossman School of Medicine Institutional Review Board. A waiver of informed consent and a waiver of the Health Information Portability Privacy act were granted.

RESULTS

Patients taking zinc sulphate in addition to hydroxychloroquine and azithromycin (n=411) and patients taking hydroxychloroquine and azithromycin alone (n=521) did not differ in age, race, sex, tobacco use or past medical history (Table 1). On hospital admission, vital signs differed by respiratory rate and baseline systolic blood pressure. The

FOI 26-3132 - Document 5

Table 1. Comparisons of baseline characteristics and hospital medications. Data are represented as median (IQR) or mean±SD. Sample size is reported where it differed due to lab results not tested. *P* values were calculated using two-sided *t*-test for parametric variables and Mann–Whitney U test for nonparametric continuous variables. Pearson's χ^2 test was used for categorical comparisons. $P < 0.05$ was deemed significant. Laboratory results represent the first measured value while hospitalized

	Zinc ***N*=411**	**No zinc** ***N*=521**	***P* value**
Demographics			
Age	63.19±15.18	61.83±15.97	0.0942
Female Sex	147 (35.7%)	201 (38.6%)	0.378
Race			0.428
African American	68 (16.5%)	81 (15.5%)	
White	189 (46.0%	244 (46.8%)	
Asian	30 (7.3%)	30 (5.8%)	
Other	97 (23.6%)	142 (27.2%)	
Multiracial/Unknown	27 (6.6%)	24 (4.6%)	
History			
Tobacco use			0.142
Never or Unknown	306 (74.5%)	382 (73.3%)	
Former	76 (18.5%)	115 (22.1%)	
Current	29 (7.1%)	24 (4.6%)	
Any cardiovascular condition	182 (44.3%)	248 (47.6%)	0.313
Hypertension	154 (37.5%)	208 (39.9%)	0.445
Hyperlipidemia	99 (24.1%)	148 (28.4%)	0.138
Coronary Artery Disease	36 (8.8%)	41 (7.9%)	0.624
Heart Failure	26 (6.3%)	22 (4.2%)	0.149
Asthma or COPD	50 (12.2%)	56 (10.7%)	0.499
Diabetes	105 (25.5%)	130 (25.0%)	0.835
Malignancy	23 (5.6%)	33 (6.3%)	0.638
Transplant	3 (0.7%)	2 (0.4%)	0.473
Chronic Kidney Disease	47 (11.4%)	44 (8.4%)	0.127
BMI kg/m^2	29.17 (25.8–33.42)	29.29 (25.77–33.2)	0.8611
Admission Characteristics			
Oxygen saturation at presentation	94 (91–96)*	94 (91–96)†	0.1729
Respiratory Rate, respirations per minute	20 (19–24)	20 (18–24)	**0.0460**
Pulse, beats per minute	97.66±18.61	99.40±19.82	0.0858
Baseline Systolic BP, mmHg	134.83±20.84	132.41±21.87	**0.0435**
Baseline Diastolic BP, mmHg	76.66±12.62	76.59±14.22	0.4670
Temperature, degrees Celsius	37.65±0.82	37.72±0.94	0.1354
White blood cell count 10^3/ul	6.9 (5.1–9.0) *N*=400	6.9 (5.1–9.3) *N*=500	0.5994
Absolute neutrophil count, 10^3/ul	5.15 (3.6–7.05) *N*=388	5.4 (3.8–7.5) *N*=488	0.0838

Continued

 FOI 26-3132 - Document 5

Table 1. Continued

	Zinc *N*=411	No zinc *N*=521	*P* value
Absolute lymphocyte count, 10^3/ul	1 (0.7–1.3) *N*=388	0.9 (0.6–1.3) *N*=482	**0.0180**
Ferritin, ng ml^{-1}	739 (379–1528) *N*=397	658 (336.2–1279) *N*=473	0.1304
D-Dimer, ng ml^{-1}	341 (214–565) *N*=384	334 (215–587) *N*=435	0.7531
Troponin, ng ml^{-1}	0.01 (0.01–0.02) *N*=389	0.015 (0.01–0.02) *N*=467	**0.0111**
Creatine Phosphokinase, U l^{-1}	140 (68–330) *N*=343	151.5 (69.5–398.5) *N*=344	0.4371
Procalcitonin, ng l^{-1}	0.12 (0.05–0.25) *N*=395	0.12 (0.06–0.43) *N*=478	**0.0493**
Creatinine, mg l^{-1}	0.97 (0.8–1.34) *N*=400	0.99 (0.8–1.27) *N*=499	0.4140
C-Reactive Protein, mg l^{-1}	104.95 (51.1–158.69) *N*=398	108.13 (53–157.11) *N*=480	0.9586
Medications recorded during hospitalization			
NSAID	53 (12.9%)	74 (14.2%)	0.563
Anticoagulant	402 (97.8%)	511 (98.1%)	0.772
ACE inhibitor or ARB	138 (33.6%	175 (33.7%)	0.997
Beta Blocker	91 (22.1%)	132 (25.3%)	0.256
Calcium Channel Blocker	89 (21.7%)	104 (20.0%)	0.527
Corticosteroid	40 (9.7%)	47 (9.0%)	0.711

*Measured on supplemental oxygen for 86.4%
†Measured on supplemental oxygen for 83.1%.

first laboratory measurements of inflammatory markers including white blood cell count, absolute neutrophil count, ferritin, D-dimer, creatine phosphokinase, creatinine and C-reactive protein did not differ between groups. Patients treated with zinc sulphate had higher baseline absolute lymphocyte counts [median (IQR), zinc: 1 (0.7–1.3) vs. no zinc: 0.9(0.6–1.3), *P* value: 0.0180] while patients who did not receive zinc had higher baseline troponin [0.01 (0.01–0.02) vs. 0.015 (0.01–0.02), *P* value: 0.0111] and procalcitonin [0.12 (0.05–0.25) vs 0.12 (0.06–0.43), *P* value: 0.0493] (Table 1).

In univariate analysis, the addition of zinc sulphate to hydroxychloroquine and azithromycin was not associated with a decrease in length of hospital stay, duration of mechanical ventilation, maximum oxygen flow rate, average oxygen flow rate, average fraction of inspired oxygen or maximum fraction of inspired oxygen during hospitalization (Table 2). In bivariate logistic regression analysis, the addition of zinc sulphate was associated with decreased mortality or transition to hospice (OR 0.511, 95% CI 0.359–0.726), need for ICU (OR 0.545, 95% CI 0.362–0.821) and need for invasive ventilation (OR 0.562, 95% CI 0.354–0.891) (Table 3). However, after excluding all non-critically ill patients admitted to the intensive care unit, zinc sulphate no longer was found to be associated with a decrease in mortality (Table 3). Thus, this association was driven by patients who did not receive ICU care (OR 0.492, 95% CI 0.303–0.799). We also found that the addition of zinc sulphate was associated with likelihood of discharge to home in univariate analysis (OR 1.56, 95% CI 1.16–2.10) (Table 3). We performed a logistic regression model to account for the time-period when the addition of zinc sulphate to hydroxychloroquine plus azithromycin became utilized at NYULH. After adjusting for this date (25 March), we still found an association for likelihood of discharge to home (OR 1.53, 95% CI 1.12–2.09) and decreased mortality or transition to hospice however the other associations were no longer significant (Table 3). The decrease in mortality or transition to hospice was most striking when considering only patients who were not admitted to the ICU (OR 0.449, *P* value: 0.002) (Table 3).

 FOI 26-3132 - Document 5

Table 2. Comparisons of continuous hospital outcomes. Data are represented median (IQR) and as mean±SD. Sample size is reported for each variable tested. β coefficients and *P* values were calculated using linear regression. *N* was specified for each comparison. *P*<0.05 was deemed significant

	Zinc	No zinc	β coefficient	*P* value
Length of ospital stay (in days)*	6 (4–9) *N*=411	6 (3–9) *N*=521	0.015	0.646
Duration of mechanical* ventilation (in days)	5 (3–8) *N*=33	5 (3–9) *N*=86	0.040	0.667
ICU duration (in days)*	4.85 (1.97–7.94) *N*=38	5.54 (2.65–9.32) *N*=82	−0.062	0.504
Oxygen flow rate maximum*	6 (3–15) *N*=353	6 (3–15) *N*=426	−0.015	0.679
Oxygen flow rate average*	3.05 (2.1–6.3) *N*=353	3.5 (2.5–7.5) *N*=426	−0.062	0.082
Fraction of inspired oxygen, average	61.52±32.03 *N*=107	65.26±34.48 *N*=117	−0.056	0.402
Fraction of inspired oxygen, maximum	74.94±35.75 *N*=107	71.98±35.85 *N*=117	0.041	0.538

*Variables were log transformed for regression analysis. Intensive care unit is abbreviated to ICU.

DISCUSSION

While practicing at the epicentre of the pandemic in the United States, we were faced with unprecedented challenges of adopting investigational therapies quickly into clinical practice. Initially, antiviral options at our institution consisted of clinician preference for either ritonavir/lopinavir or hydroxychloroquine plus azithromycin. After the findings of ritonavir/lopinavir, we noticed an increase in the use of hydroxychloroquine plus azithromycin [16]. Our providers within the infectious diseases division, clinical pharmacy and hospitalists discussed the use of zinc sulphate as an addition to hydroxychloroquine, based on the potential synergistic mechanism, and low risk of harm associated with this therapy.

There has been significant interest in the use of zinc sulphate to treat and prevent COVID-19 infection and its use is being considered in several trials (NCT04326725, NCT04377646, NCT04370782, NCT04373733, NCT04351490) [9, 17, 18]. To our knowledge, we provide the first *in vivo* evidence on the efficacy of zinc sulphate in addition to hydroxychloroquine in COVID-19 patients. The main finding of this study is that the addition of zinc sulphate to hydroxychloroquine and

Table 3. Comparison of categorical hospital outcomes. Data are represented as *N* (%). *P* values were calculated using logistic regression or multivariate logistic regression adjusting for patient admission after 25 March as a categorical variable. *P*<0.05 was deemed significant. *N* was specified for subgroup analyses. Intensive care unit is abbreviated to ICU

	Discharged home	Needed ICU	Needed invasive Ventilation	Expired/hospice	Expired/hospice*	Expired/hospice†
Zinc *N*=411	317 (77.1%)	38 (9.2%)	29 (7.1%)	54 (13.1%)	28 (73.6%) *N*=38	26 (6.9%) *N*=373
No Zinc *N*=521	356 (68.3%)	82 (15.7%)	62 (11.9%)	119 (22.8%)	61 (74.4%) *N*=82	58 (13.2%) *N*=439
Odds Ratio	1.56	0.545	0.562	0.511	0.964	0.492
95% Confidence Interval	1.16–2.10	0.362–0.821	0.354–0.891	0.359–0.726	0.401–2.31	0.303–0.799
***P* value**	**0.003**	**0.004**	**0.014**	**<0.0001**	0.934	**0.004**
Adjusted Odds Ratio	1.53	0.733	0.804	0.559	1.03	0.449
Adjusted 95% Confidence Interval	1.12–2.09	0.471–1.14	0.487–1.33	0.385–0.811	0.404–2.64	0.271–0.744
Adjusted *P* value	**0.008**	0.168	0.396	**0.002**	0.947	**0.002**

*After excluding all non-ICU patients.
†After excluding all ICU patients.

 FOI 26-3132 - Document 5

azithromycin was found to be associated with a decrease in mortality or transition to hospice among patients who did not require ICU level of care, but this association was not seen for patients who were treated in the ICU. This result may reflect one of the proposed mechanisms by which zinc sulphate may provide protection against COVID-19. Zinc has been shown to reduce SARS-CoV RNA-dependent RNA polymerase activity *in vitro* [14]. As such, zinc may have a role in preventing the virus from progressing to severe disease, but once the aberrant production of systemic immune mediators is initiated, known as the cytokine storm, the addition of zinc may no longer be effective [19]. To further examine the ability of zinc to prevent viral replication and disease progression, future studies should measure viral RNA levels in clinical specimens before and after zinc administration. Our findings suggest a potential protective effect of zinc, potentially enhanced by a therapeutic synergistic mechanism of zinc sulphate with hydroxychloroquine, if used early on in presentation with COVID-19.

After adjusting for the timing of zinc sulphate treatment, the negative associations between zinc and the need for ICU and invasive ventilation were no longer significant but we did still observe a trend. This observation may be because patients with COVID-19 were initially sent to the ICU quicker, but as time went on and resources became more limited, clinicians began treating COVID-19 patients on general medicine floors for longer periods of time before escalating to the ICU. Future studies are needed to confirm or refute the hypothesis that the addition of zinc sulphate to a zinc ionophore such as hydroxychloroquine may reduce the need for ICU care in patients with COVID-19.

This study has several limitations. First, this was an observational retrospective analysis that could be impacted by confounding variables. This is well demonstrated by the analyses adjusting for the difference in timing between the patients who did not receive zinc and those who did. In addition, we do not know whether the observed added benefit of zinc sulphate to hydroxychloroquine and azithromycin on mortality would have been seen in patients who took zinc sulphate alone or in combination with just one of those medications since few patients at our hospitals received zinc sulphate as stand-alone therapy. The optimal dose and formulation of zinc supplementation necessary to inhibit RNA-dependent RNA polymerase activity of coronaviruses also remains unknown. Early reports suggested higher doses may be necessary, ranging from 50 to 150 mg elemental zinc/day, and such doses have been proved to be safe for short periods of time when used for other viruses or in improving immune reconstitution [20–23]. In addition, although other formulations of zinc supplementation exist, in the form of acetate or gluconate, our hospital formulary had sulphate, which generally has a higher elemental amount of zinc per tablet. Therefore, further investigation is required to determine whether sulphate is the preferred formulation when used to treat coronaviruses, or if another formulation may be better tolerated. Given the recent concern regarding the potential for side effects associated with hydroxychloroquine, future studies should examine whether zinc sulphate would provide benefit as a stand-alone therapy or in combination with another zinc ionophore. In addition, as zinc is hypothesized to inhibit viral RNA polymerase, future studies are needed to determine if zinc synergistically acts with other antiviral medications. We also do not have data on the time at which the patients included in the study initiated therapy with hydroxychloroquine, azithromycin and zinc. Those drugs would have been started at the same time as a combination therapy, but the point in clinical disease at which patients received those medications could have differed between our two groups. Finally, the cohorts were identified based on medications ordered rather than confirmed administration, which may bias findings towards favouring equipoise between the two groups.

CONCLUSION

Zinc sulphate added to hydroxychloroquine and azithromycin associates with a decrease in mortality or transfer to hospice among patients who do not require ICU level of care and an increased likelihood to be discharged directly home from the hospital. In light of study limitations, this study alone is not sufficient to guide clinical practice. Rather, these findings suggest a potential role for zinc sulphate in COVID-19 patients and support the initiation of future randomized clinical trials investigating zinc sulphate against COVID-19.

Funding information
The authors received no specific grant from any funding agency.

Acknowledgement
The authors thank Mark Mulligan, Andrew Admon, Mary Grace Fitzmaurice, Brian Bosworth, Robert Cerfolio, Steven Chatfield, Thomas Doonan, Fritz Francois, Robert Grossman, Leora Horwitz, Juan Peralta, Katie Tobin and Daniel Widawsky for their operational and technical support. We also thank the thousands of NYU Langone Health employees who have cared for these patients.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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EClinicalMedicine 000 (2021) 100720

Contents lists available at ScienceDirect

EClinicalMedicine

journal homepage: https://www.journals.elsevier.com/eclinicalmedicine

Research Paper

The effect of early treatment with ivermectin on viral load, symptoms and humoral response in patients with non-severe COVID-19: A pilot, double-blind, placebo-controlled, randomized clinical trial

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ARTICLE INFO

Article History:
Received 1 December 2020
Revised 28 December 2020
Accepted 28 December 2020
Available online xxx

Keywords:
Ivermectin
SARS-CoV-2
COVID-19
Anosmia
Hyposmia

ABSTRACT

Background: Ivermectin inhibits the replication of SARS-CoV-2 in vitro at concentrations not readily achievable with currently approved doses. There is limited evidence to support its clinical use in COVID-19 patients. We conducted a Pilot, randomized, double-blind, placebo-controlled trial to evaluate the efficacy of a single dose of ivermectin reduce the transmission of SARS-CoV-2 when administered early after disease onset.

Methods: Consecutive patients with non-severe COVID-19 and no risk factors for complicated disease attending the emergency room of the Clínica Universidad de Navarra between July 31, 2020 and September 11, 2020 were enrolled. All enrollments occurred within 72 h of onset of fever or cough. Patients were randomized 1:1 to receive ivermectin, 400 mcg/kg, single dose (n = 12) or placebo (n = 12). The primary outcome measure was the proportion of patients with detectable SARS-CoV-2 RNA by PCR from nasopharyngeal swab at day 7 post-treatment. The primary outcome was supported by determination of the viral load and infectivity of each sample. The differences between ivermectin and placebo were calculated using Fisher's exact test and presented as a relative risk ratio. This study is registered at ClinicalTrials.gov: NCT04390022.

Findings: All patients recruited completed the trial (median age, 26 [IQR 19–36 in the ivermectin and 21–44 in the controls] years; 12 [50%] women; 100% had symptoms at recruitment, 70% reported headache, 62% reported fever, 50% reported general malaise and 25% reported cough). At day 7, there was no difference in the proportion of PCR positive patients (RR 0·92, 95% CI: 0·77–1·09, p = 1·0). The ivermectin group had non-statistically significant lower viral loads at day 4 (p = 0·24 for gene E; p = 0·18 for gene N) and day 7 (p = 0·16 for gene E; p = 0·18 for gene N) post treatment as well as lower IgG titers at day 21 post treatment (p = 0·24). Patients in the ivermectin group recovered earlier from hyposmia/anosmia (76 vs 158 patient-days; $p < 0.001$).

Interpretation: Among patients with non-severe COVID-19 and no risk factors for severe disease receiving a single 400 mcg/kg dose of ivermectin within 72 h of fever or cough onset there was no difference in the

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https://doi.org/10.1016/j.eclinm.2020.100720
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Please cite this article as: C. Chaccour et al., The effect of early treatment with ivermectin on viral load, symptoms and humoral response in patients with non-severe COVID-19: A pilot, double-blind, placebo-controlled, randomized clinical trial, EClinicalMedicine (2021), https://doi.org/10.1016/j.eclinm.2020.100720

proportion of PCR positives. There was however a marked reduction of self-reported anosmia/hyposmia, a reduction of cough and a tendency to lower viral loads and lower IgG titers which warrants assessment in larger trials.

Funding: ISGlobal, Barcelona Institute for Global Health and Clínica Universidad de Navarra.

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Research in context

Evidence before this study

Ivermectin is a broad spectrum antiparasitic drug with known antiviral properties. On April 3, 2020, Caly et al. published evidence from in vitro experiments showing that ivermectin can inhibit the replication of SARS-CoV-2 at micromolar concentrations. This has torn the scientific community into two opposing views, one group calling for avoiding investment and effort in a drug likely to fail clinical trials, and another group calling for rapid scale-up even in the absence of proven safety and efficacy for the potential COVID-19 indication. Since April 2020, there has been an abundance of observational trials, case series and ecological analyses suggesting a potential efficacy of ivermectin against COVID-19. Yet very few reports of rigorously conducted randomized controlled clinical trials. Emerging evidence from studies conducted in Bangladesh and Argentina points towards a potential clinical use.

Added value of this study

This pilot, randomized, placebo-controlled, double blind trial failed to show a reduction in the proportion of PCR-positive patients seven days after ivermectin treatment; yet it shows a reduction in the self-reported anosmia/hyposmia and a (non-statistically significant) tendency to lower viral loads and lower IgG titers which presumably reflect milder disease.

Implications of all the available evidence

The positive signal found in this pilot together with emerging evidence from animal models and other clinical trials warrants the conduction of larger trials using ivermectin for the early treatment of COVID-19.

1. Introduction

As of December 21, 2020, there have been over 70 million cases and 1·6 million COVID-19 deaths worldwide [1]. Although the threshold is difficult to predict accurately [2], the spread of SARS-CoV-2 is unlikely to stop before at least 50% of the population has gained immunity, either by vaccination or recovering from a naturally-acquired infection [3]. There are now promising vaccines candidates advancing to emergency regulatory approval [4], but there is a projected delay in global access to the level required for population impact on the trajectory of the pandemic. While efforts are ongoing to develop treatment options, relatively less attention has been devoted to evaluating drug-based transmission blocking or transmission reduction strategies. These strategies would consist in administering a drug with the aim of reducing onward transmission by those infected and could serve to reduce the burden on health system and gain time until vaccines are fully tested and scaled-up.

Ivermectin is a widely used antiparasitic drug with known partial efficacy against several single-strain RNA viruses [5-7]. Caly et al. reported in vitro inhibition of SARS-CoV-2 replication using micromolar concentrations of ivermectin [8]. These findings, together with early observational evidence and ecological evidence, prompted several Latin-American countries to include ivermectin as part of the national policy for COVID-19 treatment [9].

As of December 21, 2020, there are 45 studies evaluating the efficacy of ivermectin to treat or prevent COVID-19 registered in clinicaltrials.gov, and 74 trials registered in WHO's International Clinical Trials Registry Platform (https://apps.who.int/trialsearch/) of which at least 14 are already completed. Although some observational and case control studies as well as emerging small randomized clinical trials suggest a potential utility [10-13]. Yet, there is still a dearth of robust, randomized controlled trials to appropriately inform policy decisions.

This trial was designed as a pilot to evaluate whether the maximum approved dose of ivermectin in Europe could have an impact on the transmission of SARS-CoV-2 when administered early after disease onset.

2. Methods

This was a pilot, double-blind, placebo-controlled, single-center, parallel-arm, superiority, randomized clinical trial that compared a single dose of ivermectin with placebo in patients with non-severe COVID-19 and no risk factors. The trial protocol was published [14], the last version of the protocol and statistical analysis plan are available as supplementary files. The protocol was approved by the Spanish national ethics committee for drug research (Hospital Puerta de Hierro Majadahonda) and by the Spanish Agency of Medicines and Medical Devices. All procedures were conducted in compliance with the latest revision of the Helsinki Declaration and Good Clinical Practice. All patients provided verbal informed consent at enrollment followed by written consent once their isolation was lifted in accordance to the EMA recommendations: "Guidance on the Management of Clinical Trials during the COVID-19 (Coronavirus) pandemic Version 2 (27/03/2020)" [15]. This study is registered at ClinicalTrials.gov: NCT04390022. This study was funded by ISGlobal and the Clínica Universidad de Navarra. The funding sources had no role on the design, analysis or decision to publish the results of this study.

2.1. Patients

Consecutive outpatients attending the Emergency Room of the Clínica Universidad de Navarra (Pamplona, Spain) with symptoms compatible with COVID-19, no more than 72 h of fever or cough and a positive PCR for SARS-CoV-2 were enrolled. Patients with positive IgG against SARS-CoV-2, comorbidities considered risk factors for severe disease or COVID-19 pneumonia at baseline were excluded (detailed eligibility criteria are provided in the protocol -Supplementary file-).

2.2. Study design and oversight

The trial was conducted in the Pamplona metropolitan area (Navarra, Spain). Patients were enrolled between July 31, 2020 and September 11, 2020 and randomized in a 1:1 ratio to ivermectin (400 mcg/kg) single oral dose or placebo. The randomization sequence was computer-generated by the trial statistician using blocks of four

to ensure balance. Allocation was made by the attending investigator using opaque envelopes. The placebo tablets did not match ivermectin in appearance, therefore, in order for the clinical trial team to remain blinded, treatment was administered under direct supervision by a non-participant nurse that picked up the opaque bottles directly from the pharmacy and administered the content behind closed doors. The clinical trial team had no contact with the investigational products. There was slow recruitment due to a sharp reduction in local transmission for 10 weeks after the lockdown of March-April 2020, the protocol was amended on September 2nd to extend the inclusion criteria from 48 to a maximum of 72 h of cough or fever.

The main objective was to determine the efficacy of a single dose of ivermectin, administered to low risk, non-severe COVID-19 patients in the first 72 h after fever or cough onset to reduce onward transmission.

2.3. Clinical, laboratory and virological monitoring

Assessments on enrollment and at days 4, 7, 14, 21 and 28 post treatment included: general symptoms report, physical examination (including respiratory rate, blood oxygen saturation and chest auscultation) and adverse events. All patients were asked to complete a daily online diary of symptoms from day 1 to 28 post treatment. On enrollment, as well as on days 7 and 14 blood samples were obtained to assess full blood count, C reactive protein, procalcitonin, ferritin, creatinine phosphokinase, lactic dehydrogenase, troponin T, D dimer, IL-6, and renal function.

A nasopharyngeal swab for SARS-CoV-2 PCR was taken at enrollment and on days 4, 7, 14 and 21 post treatment. For consistency, these samples were collected by three clinicians using the same technique. All samples were processed by PCR for genes N and E of SARS-CoV-2 (Real Time PCR SARS-CoV-2, Vircell SLU, Granada, Spain). For every sample, the viral load was calculated using standard reference curve (EDX Sars-Cov-2, Exact Diagnostics LLC, Fort Worth Texas). Additionally, all samples from day 4 post treatment were cultured in Vero cells for 7 days, after which the cytopathic effect was assessed and PCR conducted on the harvested cell-free supernatant. If the PCR from the supernatant was positive at day 4, the procedure was repeated on the samples of that patient for day 7. A semi-quantitative serology for IgG against SARS-CoV-2 (COVID-19 VIRCLIA IgG monotest, Vircell SLU, Granada, Spain) was done on samples from all patients on day 21 post-treatment.

2.4. Outcome measures

The primary outcome measure was the proportion of patients with detectable SARS-CoV-2 RNA by PCR from nasopharyngeal swab at day 7 post-treatment.

Relevant pre-specified secondary outcomes included viral load at days 4, 7, 14 and 21 post treatment; proportion of patients with symptoms (particularly fever and cough) at days 4, 7, 14 and 21 post-treatment as well as proportion of patients progressing to severe disease or death during the trial; proportion of patients with seroconversion at day 21 post-treatment and proportion of drug-related adverse events.

2.5. Sample size justification

In COVID-19, viral load peaks right before or at symptom onset [16,17] and most secondary cases occur prior to day five after symptoms [18]. This pilot was designed to assess the use of ivermectin to reduce transmission. With the objective to reduce onward transmission, a robust effect size in the proportion of PCR positives at day seven after treatment would be needed to have a public health impact. A reduction of at least 50% in the proportion of positives was considered of potential value.

The sample size was based in the comparison of two proportions and calculated to have 80% power at a 5% significance level to detect a 50% reduction (100 vs 50%) in the proportion of participants with positive PCR at day 7 post-treatment. The 100% PCR positivity figure at day 7 is based on the experience with COVID-19 outpatients at the Clínica Universidad de Navarra during the first wave of March-May 2020. The infectivity outcome was supported by assessing changes in viral load and infectivity in cell cultures.

2.6. Statistical analysis

Descriptive analyses used frequency and percentage (based on the non-missing sample size) for qualitative variables and median, interquartile range and n (non-missing sample size) for quantitative variables.

For the primary objective, the proportion of participants with positive PCR at day seven post treatment was calculated. Proportions were compared between study arms using Fisher's exact test and presented as a relative risk ratio (RR) with their corresponding 95% confidence interval (CI). In the analysis of the symptoms reported by patients (symptom diary), missing data was carried over from the last data available. Significance was set at 0.05. The analysis was carried out using Stata (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC).

Boxplots and bar plots were produced for the description of quantitative and qualitative variables, respectively. For figure readability, viral load values were log-transformed. Graphs were produced in R version 4.0.2 (R Core Team, R: A Language and Environment for Statistical Computing, Vienna, Austria: R Foundation for Statistical Computing, 2020) with the package ggplot2 (H. Wickham, ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag New York, 2016.).

Viral load data were synchronized prior to analysis by accounting for days since onset of any symptoms and, since the day of infection was not known, an average incubation time of 5 days was assumed [19]. Peak viral load (Cmax) and time to peak viral load (Tmax) were determined directly from the profiles. Area under the viral load curve was calculated using the trapezoidal rule from assumed time of infection to last sample (AUCobs). Duration of time above a cycle threshold (Ct) of 35 was derived directly from profiles or linearly extrapolated profiles if the last recorded Ct value was not below the threshold.

2.7. Post hoc analyses

The median viral load at all sampling times and median IgG titers between study groups were compared using a Wilcoxon rank-sum test.

The effect of study arm on the presence of symptoms was estimated using mixed effect logistic regression models with subject as a random intercept. These models are adjusted by day of follow up (as symptoms are expected to disappear over time) and duration of symptoms before enrolment (as a proxy of disease onset). To assess the potential effect of study arm on symptom progression, the interaction between study arm and day of follow up was also included in the models. Three models were studied for those outcomes for which differences between treatments were observed: any symptom, anosmia or hyposmia, and cough. Additionally, the observed effect of ivermectin on anosmia/hyposmia was assessed in a sub-analysis by sex.

The adipose weight of participants in the ivermectin group was calculated with the method described by Gomez-Ambrosi et al. [20]. This information was used to estimate the ivermectin dose per

adipose-weight received and plotted against the last day of reported anosmia/hyposmia.

3. Results

3.1. Patient characteristics

Of 94 patients assessed, 50 did not meet eligibility criteria, 20 declined to participate and 24 were randomized. All randomized patients received the corresponding study product and completed 28 days of follow-up (Fig. 1). The baseline characteristics of patients in both groups are presented in Table 1.

There was a higher proportion of females in the placebo group (58 vs 42%). Demographics and baseline disease characteristics of participants in both groups are presented in Table 1. Overall, 66% of the patients presented with perceived or objective fever, 25% presented with cough, 70% presented with headache and 58% presented with myalgia or general malaise with no remarkable differences between groups. The median earliest start of any symptom before treatment was 24 h for the ivermectin group (interquartile range, 24–48 h) and 48 h for the placebo group (interquartile range, 36–48 h). At baseline, there were no differences in vital signs, inflammatory markers or full blood count between the groups (Table 1).

3.2. Primary endpoint

There was no difference in the proportion of PCR positive patients at day 7 post treatment, 12/12 (100%) patients had a positive PCR for gene N in both groups. For gene E, 11/12 (91%) in the ivermectin and 12/12 (100%) in the placebo group had a positive PCR (RR 0·92, 95% CI: 0·77–1 0·09, $p = 1·0$).

3.3. Viral load

Genes E and N had comparable results at all time points. Patients in both study groups had similar viral load before treatment with median and interquartile range for genes E and N in the same orders of magnitude (Fig. 2 and Table S1). Although there was a consistent overlap in interquartile ranges and full ranges at all points, the median viral load for both genes was lower at days 4 and 7 post

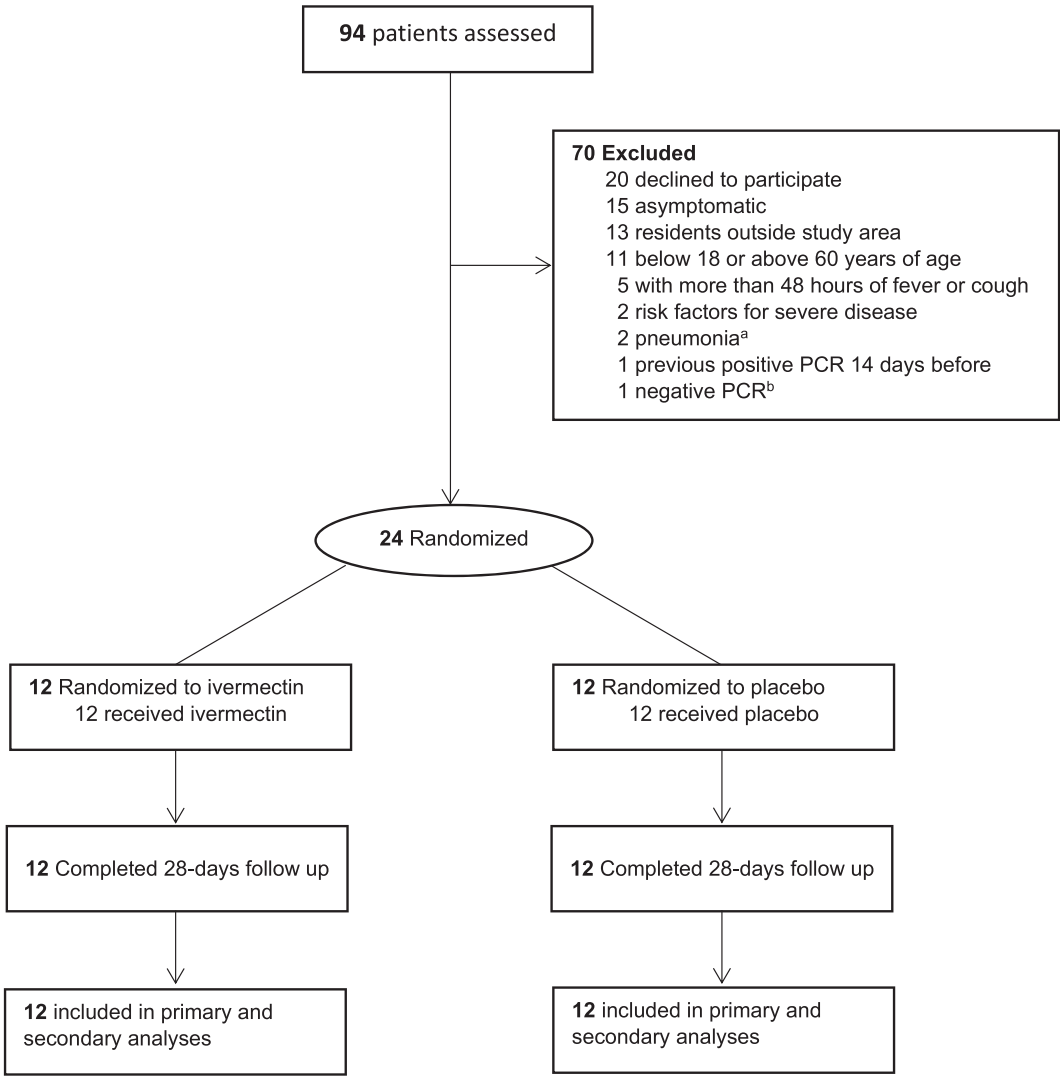


Fig. 1. Enrollment and patient flow. [a] One presented with pneumonia in the ER and one had a compatible X-ray during screening. [b] Formally screened based on epidemiological and clinical suspicion but had a negative PCR.

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Table 1
Baseline characteristics of patients by group.

	Ivermectin (n = 12)	Placebo (n = 12)
Age, median (IQR)[range] (years)	26 (19–36) [18–54]	26 (21–44) [18–54]
Sex, No. (%)		
Female	5 (42%)	7 (58%)
Male	7 (58%)	5 (42%)
Body mass index, median (IQR) [range] kg/m^2	23·5 (19·6–27·8) [18·6–29· 9]	22·9 (21·0–24·8) [19·3–29·9]
Symptoms		
Any, No. (%)	12 (100%)	12 (100%)
Fever, No. (%)	7 (58%)	9 (75%)
Cough, No. (%)	4 (33%)	2 (17%)
Headache, No. (%)	7 (58%)	10 (83%)
Myalgia/general malaise, No. (%)	8 (67%)	6 (50%)
Earliest start of any symptom[a], median, (IQR) [range]	24 (24–48) [18–120]	48 (36–48) [24–72]
Earliest start of fever[a]*, No, median. (IQR) [range]	24 (12–24) [12–24], n = 7	24 (24–48) [4–48], n = 9
Earliest start of cough[a], No, median. (IQR) [range]	24 (16–36) [8–48], n = 4	10 (8–12) [8–12], n = 2
Vital signs		
Systolic Blood pressure, median. (IQR), mmHg	114 (113–117)	129 (116–134)[b]
Diastolic blood pressure, median. (IQR), mmHg	76 (72–80)	79 (77–85)
Heart rate, median (IQR), bpm	83 (77–99)	90 (81–100)
Respiratory rate, median (IQR), bpm	14 (12–17)	14 (12–15)
Temperature, median (IQR), °C	36·8 (36·4–37·0)	36·9 (36·5–37·0)
Oxygen saturation, median (IQR),%	97 (96–98)	98 (97–100)
Viral load		
Gene E, No. (IQR), copies/ml	1·7·10^7 (5·9·10^6- 3·9·10^8)	2·7·10^7 (8·3·10^5- 4·2·10^8)
Gene N, No. (IQR), copies/ml	3·7·10^8 (1·8·10^7- 9·3·10^9)	3·3·10^8 (5·8·10^7- 6·7·10^9)
Inflammatory markers		
CRP, median (IQR), mg/dL [normal value]	0·3 (0·2–0·8) [<0·5]	0·3 (0·2–0·6) [<0·5]
Ferritin, median (IQR), mg/dL [normal value]	165·0 (95·8 - 241·3) [30–400]	156·1 (103·1–223·0) [30–400]
IL-6, median (IQR), pg/mL [normal value]	6·5 (5·1 - 9·6) [<7]	4·5 (3·0–6·5) [<7]
D-Dimer, median (IQR), ng/mL [normal value]	295 (270–420) [150–500]	280 (270–315) [150–500]
Full blood count		
Red blood cells, median (IQR), 10^{12}/L	5·05 (4·62–5·55)	5·07 (4·67– 5·45)
Hemoglobin, median (IQR), g/dL	15·3 (13·8–16·0)	15·2 (13·7–15·8)
Platelets, median (IQR), 10^9/L	194 (167–216)	205 (179–247)
White blood cells, median (IQR), 10^9/L	4·7 (4·3–6·3)	4·4 (3·7–5·9)
Neutrophils, median (IQR),%	52·4 (45·6–65·1)	53·4 (43·9–62·2)
Lymphocytes, median (IQR),%	29·5 (18·5- 7·9)	28·7 (20·8–39·9)

[a] Hours before dosing
[b] The slightly higher median systolic blood pressure in the placebo group at baseline was not seen in subsequent study visits and was judged as non-clinically significant, see table S3 for the evolution of all vital signs throughout the study, *Reported or measured fever. IQR: interquartile range

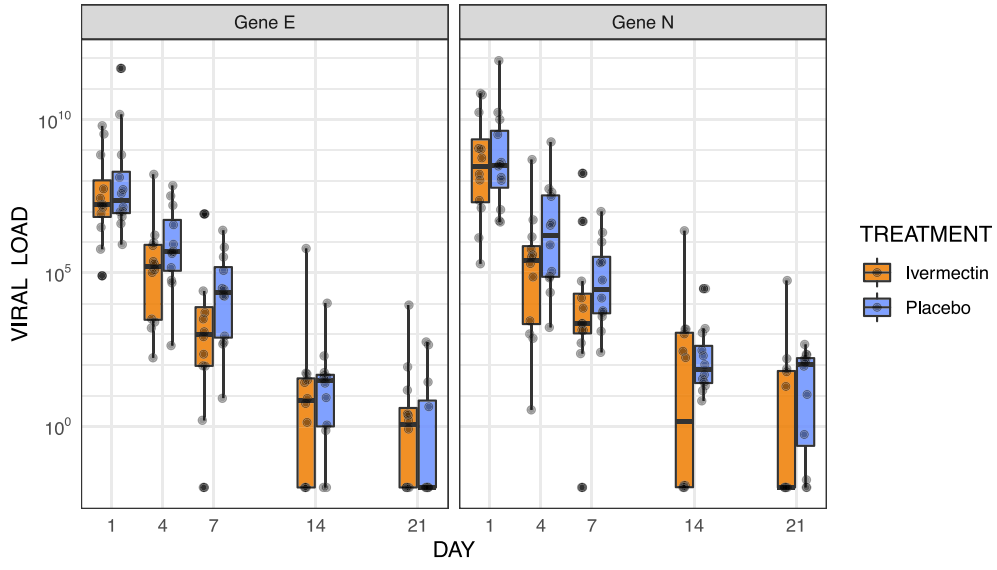


Fig. 2. Viral load evolution by study arm. Viral load values were log-transformed. The boxes show the interquartile range. Dots represent each individual value.

treatment in the ivermectin group with differences increasing from 3-fold lower at day 4 (p = 0·24 for gene E; p = 0·18 for gene N) to around 18-fold lower at day 7 (p = 0·16 for gene E; p = 0·18 for gene N) (Fig. 2 and Table S1). A similar tendency remained for the viral load at days 14 and 21, with values from patients in the ivermectin group consistently lower for at least one of the genes, the difference was not statistically significant at any single point (Fig. 2 and Table S1). The values of cycle thresholds had a very similar behavior (Figure S1). Summary statistics for viral kinetics are provided in Table S2.

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3.4. Viral culture

At day 4 post-treatment, 7/12 samples in the ivermectin and 5/12 samples in the placebo group effectively replicated Vero cell culture; the median Ct values of replicating samples were 23·3 and 23·8 for genes N and E respectively, while the median Ct values of non-replicating samples were 27·6 and 27·9 for genes N and E respectively. By day 7 post treatment only 1/6 in the ivermectin (one previously positive sample was lost) and 1/5 in the placebo group replicated in the cell culture; the median Ct values of replicating samples were 25·1 and 26·0 for genes N and E respectively, while the median Ct values of non-replicating samples were 30·8 and 32·0 for genes N and E respectively.

3.5. Symptoms

There was good compliance with the daily online questionnaire with 282 patient-days reports (84%) and 295 patient-days reports (88%) in the ivermectin and placebo group respectively (Fig. S2).

Patients in the ivermectin group reported fewer patient-days of any symptoms than those in the placebo group (171 vs 255 patient-days). This difference is mostly driven by two symptoms, anosmia/hyposmia and cough. Patients in the ivermectin group reported 50% less anosmia/hyposmia than those in the placebo group (76 vs 158 patient-days of anosmia/hyposmia). The ivermectin group also reported 30% less cough (68 vs 97 patient-days of cough) (Fig. 3).

There were no major differences between ivermectin and placebo in the reported patient-days of fever (12 vs 12), general malaise (51 vs 61), headache (34 vs 38), or nasal congestion (91 vs 97). With much lower magnitudes, the ivermectin group reported 3.5-fold more patient-days of gastrointestinal symptoms (21 vs 6) and 5-fold less shortness of breath (3 vs 15) (Fig. S3).

No patient from either group progressed to severe disease.

3.6. Serology

All patients in both groups seroconverted by day 21 post treatment. Patients in the ivermectin group had a lower median of IgG titers (Index 4·7, interquartile range [3·5–8·9]) than those in the placebo group (Index 7·5, interquartile range [4·2–9·3]) (p = 0·24 by Wilcoxon rank-sum test) (Fig. 4).

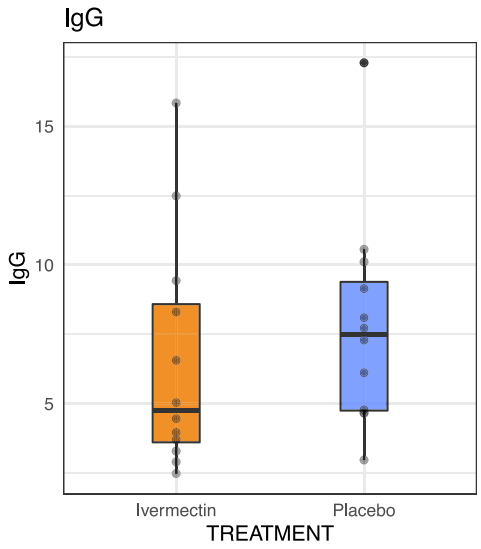


Fig. 4. IgG titers by study arm. The boxes show the interquartile range. Dots represent each individual value (p = 0·24, Wilcoxon rank-sum test).

3.7. Safety

All patients completed the follow up period of 28 days. There were 15 adverse events (7 in the ivermectin and 8 in the placebo group) experienced by 10 patients (5 in the ivermectin and 5 in the placebo group). There were no severe adverse events.

The online diary of symptoms included questions about ivermectin-specific adverse events. There were no differences in the reported patient-days between the ivermectin and the placebo group for confusion (1 vs 0), drowsiness (0 vs 0), or pruritus (0 vs 3). Patients in the ivermectin group reported more patient-days of dizziness (7 vs 1) and blurred vision (24 vs 1), with this last value driven by a single patient in the ivermectin group reporting blurred vision on days 2—28, further evaluation suggested previously undiagnosed presbyopia (Fig. S4).

There were no major differences in the evolution of vital signs (Table S3), inflammatory markers (C reactive protein, procalcitonin, ferritin and IL-6) and rest of laboratory parameters of patients in each group (Table S4).

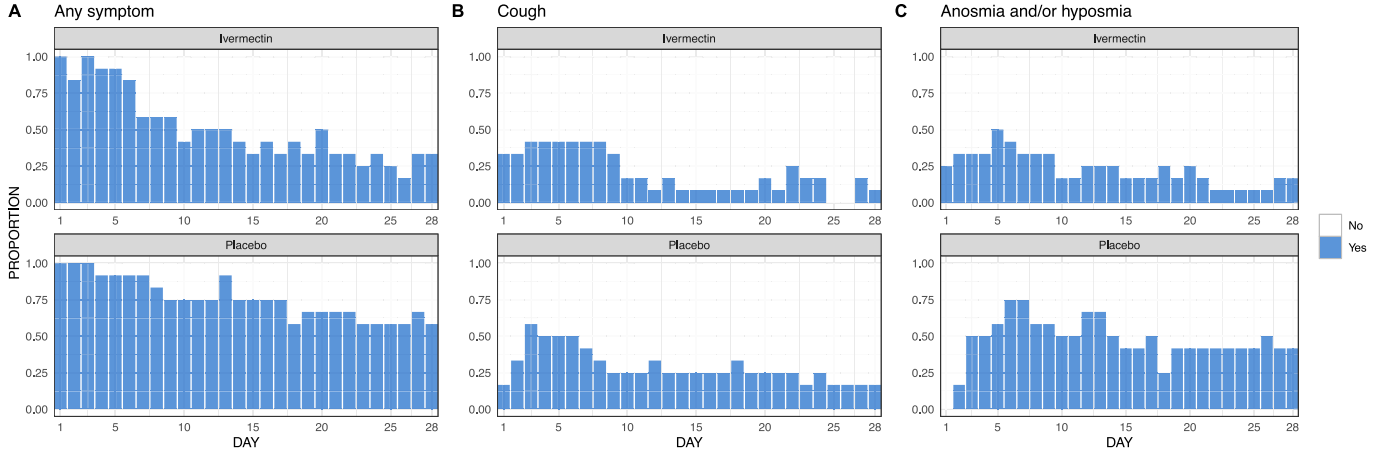


Fig. 3. Daily proportion of any self-reported symptoms, self-reported cough and self-reported anosmia/hyposmia by study arm. Each graph represents the daily proportion of individuals (n/N) who suffered from each symptom in the corresponding study arm for a 28 day follow up. Missing answers were replaced by the value in the immediately preceding day.

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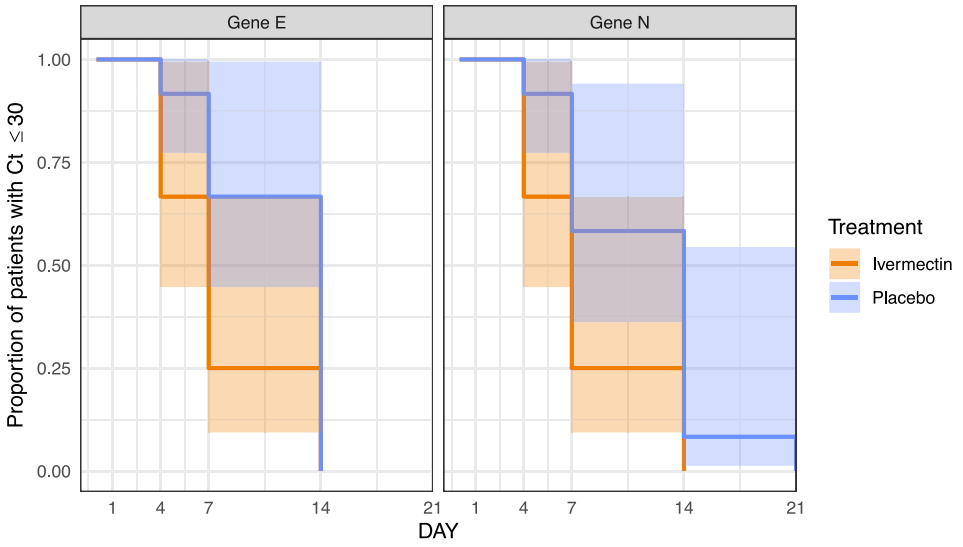


Fig. 5. Kaplan-Meier curves for viral load. A survival threshold of Ct ≥ 30 was used. Log-Rank test yielded significance for the difference in gene E (p = 0·0358) and borderline significance for the difference in gene N (p = 0·0550).

3.8. Post hoc analyses

Given that the main objective of the trial was to explore a reduction in onward transmission of the virus and that the viral cultures of samples from day 7 showed replicative virus only in samples with Ct values below 30, Kaplan-Meier curves were drawn and survival analysis conducted with log-rank test using a survival threshold of Ct ≥ 30. This analysis shows a statistically-significant difference for gene E (p = 0·035, Log-rank test) and borderline significance for gene N (p = 0·055, Log-rank test). The curves are presented in Fig. 5.

In the logistic regression model, a lower chance of presenting any symptoms was observed in the ivermectin arm (OR: 0·04 [95% CI: 0·00, 0·75] p = 0·032). In both arms, presence of any symptoms tended to diminish over time (OR (95% CI): 0·80 (0·74, 0·86) and 0·81 (0·77, 0·85), for placebo and ivermectin respectively). This downward trend was similar in both study groups (p = 0·687). With regards to the presence of cough, we did not find differences between study groups (p = 0·575) nor in the existing trend to diminish over time (p = 0·373). However, differences were observed for the presence of anosmia/hyposmia: for patients in the placebo group, there was no trend in time in the presence of anosmia (OR (95%CI): 0·99 (0·95, 1·02) p = 0·459). Conversely, patients in the ivermectin group showed a significant decrease (OR (95%CI): 0·90 (0·85, 0 0·94) p < 0·001) (Fig. S5).

The overall effect of ivermectin on anosmia/hyposmia was mainly driven by male patients (20 vs 76 patient-days of anosmia/hyposmia in the ivermectin and placebo groups respectively) in sharp contrast with female patients (56 vs 81 patient-days of anosmia/hyposmia in the ivermectin and placebo groups respectively) (Fig. S6). A sensitivity analysis to assess if sex had any impact on the logistic regression models was performed. Adding this variable to the models did not change the coefficients presented above.

The participants had an adipose weight ranging from 14% to 39% of their body weight. This resulted in ivermectin doses per adipose kilo ranging from 1028 mcg/adipose-kg to 2963 mcg/adipose-kg, even if the actual doses per full body weight were achieved in the relative narrow range of 399–427 mcg/kg (Table 2). The ivermectin doses per adipose-kg were plotted against the duration of anosmia/hyposmia and the last day of reported anosmia/hyposmia with no evident pattern, a regression was not attempted given the scarcity of data (Fig. S7).

4. Discussion

In spite of its partial antiviral properties, ivermectin received limited early attention in Europe or the US as a potential drug to be repurposed against COVID-19. This was largely based on one pharmacokinetic model stressing the inability of currently approved oral doses to reach lung tissue levels at the antiviral concentrations

Table 2
Body composition and dose of ivermectin-treated participants.

Participant ID	Age years	Sex M/F	Weight kg	Height m	BMI kg/m^2	Adipose weigh %	Adipose weight* kg	3-mg tablets received tablets	Total dose mg	Dose/kg mcg/kg	Dose adipose/kg mcg/kg
SAINT-5	24	Male	65·1	1·77	20·8	0·14	9·11	9	27	415	2963
SAINT-17	22	Male	72·7	1·79	22·7	0·17	12·43	10	30	413	2413
SAINT-18	18	Female	57·0	1·75	18·6	0·21	11·97	8	24	421	2005
SAINT-19	18	Female	45·1	1·55	18·8	0·21	9·61	6	18	399	1874
SAINT-3	33	Male	71·0	1·70	24·6	0·21	15·48	10	30	423	1938
SAINT-21	18	Female	50·1	1·60	19·6	0·22	11·47	7	21	419	1830
SAINT-16	20	Female	49·2	1·59	19·5	0·23	11·37	7	21	427	1848
SAINT-9	28	Male	89·9	1·79	28·1	0·26	23·82	12	36	400	1511
SAINT-11	47	Male	85·8	1·77	27·4	0·27	23·77	12	36	420	1515
SAINT-20	29	Male	92·2	1·79	28·8	0·27	25·54	13	39	423	1527
SAINT-8	39	Male	93·6	1·77	29·9	0·30	28·27	13	39	417	1380
SAINT-13	57	Female	66·0	1·57	26·6	0·39	26·27	9	27	409	1028

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described by Caly et al., [21] even if other, peer-reviewed models predict up to 10-fold accumulation of ivermectin in target tissue [22]. There are additional reasons to avoid direct inferences from the results of in vitro experiments or pharmacokinetic models, these include the potential role of ivermectin metabolites, the potential immunomodulatory role of the drug, and questions about the virus/cell ratios and appropriateness of the Vero cellular lines used in the cultures [23].

This pilot study was designed to assess the question of whether further investments in the potential repurposing of ivermectin were warranted. As such, we aimed at generating evidence on viral kinetics, antibody response and clinical efficacy in a cohort of patients at low risk of severe disease. Without a clearly defined mechanism of action, a sole signal in any of said parameters would not suffice to justify further efforts. This pilot shows a tendency to lower viral loads in the ivermectin group, a tendency to lower IgG titers that may reflect milder disease and clinical benefit in cardinal symptoms of COVID-19 associated with tissue damage: anosmia/hyposmia and cough. These results are in line with emerging evidence from trials in Bangladesh [10,11] and Argentina [12] showing a faster viral clearance in treated participants, as well as with recent data from a SARS-CoV-2 hamster model from Institute Pasteur which also showed a marked sex dichotomy in the effect of ivermectin on anosmia/hyposmia [24].

Pending confirmation of these results, this pilot sheds some light on the potential mechanism of action of ivermectin against COVID-19. Note the trial was not powered to detect modest differences in viral load, yet a small effect is suggested when viral load was ascertained directly by PCR and indirectly using IgG titers as markers of disease severity [25,26]. Also, in this pilot ivermectin has not shortened the duration of symptoms associated with systemic inflammation such as fever or malaise, nor has it had a measurable impact on systemic inflammatory markers.

Given these findings, consideration could be given to alternative mechanisms of action different from a direct antiviral effect. One alternative explanation might be a positive allosteric modulation of the nicotinic acetylcholine receptor caused by ivermectin and leading to a downregulation of the ACE-2 receptor and viral entry into the cells of the respiratory epithelium and olfactory bulb [27].

Another mechanism through which ivermectin might influence the reversal of anosmia is by inhibiting the activation of pro-inflammatory pathways in the olfactory epithelium. Inflammation of the olfactory mucosa is thought to play a key role in the development of anosmia in SARS-CoV-2 infection [28].

Ivermectin is known to downregulate the expression of several pro-inflammatory genes, including those of IL-8, TNF-α, and cathelicidin LL-37 [29]. This effect is thought to partially explain the efficacy of ivermectin in the treatment of rosacea [29,30]. The effect on LL-37 might be particularly important, as this molecule directly influences several pro and anti-inflammatory pathways, including the stimulation of IL-18 and IL-1β production, and has a chemotactic effect for neutrophils and eosinophils [31]. This effect might be mediated by inhibiting the entrance of the vitamin D receptor (VDR) into the nucleus [32]. Ivermectin inhibits importins of the α/β family, which play a key role in the ligand-independent transportation of the VDR, a crucial step in the vitamin D-mediated expression of the hCAMP18 gene, which encodes the LL-37 precursor [33-35]. It is possible that the inhibition of importins may thus contribute to the immune regulatory effect of ivermectin, and its influence on other vitamin D-mediated pathways, supporting further studies in this area.

Albeit requiring confirmation, these results raise several important questions. If the mechanism of action of ivermectin against COVID-19 is related to a nicotinic effect, then inhibitory concentrations for this receptor (which are in the nanomolar range) could be achievable in the lung tissue for a short period of time with oral dosing and for considerably longer periods with nebulized therapy [36]. If the mechanism is immunomodulatory, then the appropriate dose and regimen should be tailored accordingly. Before considering higher or multiple dose schemes, there is also need to better understand the potential role of ivermectin's metabolites in any observed effect. Finally, given the tendency to lower IgG titers in the ivermectin group, there is need to evaluate the potential relationship between ivermectin treatment, disease severity, inflammation, viral dynamics and antibody titers; [37,38] particular attention should be paid to the long-term humoral and cellular immune responses against SARS-CoV-2 in ivermectin treated patients.

This pilot points towards a potential use of ivermectin in COVID-19 which warrants further exploration under larger trials, with clinical outcomes in patients with risk factors or more severe disease. This is of particular importance for settings with limited resources given ivermectin's low price, broad availability and scalability of manufacturing processes.

This pilot has several key limitations that warrant careful interpretation of the results. Firstly, it was designed to explore a potential signal for the use of ivermectin in COVID-19, not to provide definitive evidence on the subject, hence its small sample size. Second this pilot was restricted to subjects with non-severe disease and no risk factors in whom the treatment was provided in the first 48 h of fever or cough, this should be taken into consideration for the design of any confirmatory studies to be conducted. Additionally, the quantification of the viral load presented is intrinsically limited by heterogeneity in the samples, even if all were obtained by the same clinicians, standardization against a human epithelial cell gene would be required to ensure the viral loads are truly comparable [39].

The positive signal found in this pilot warrants the conduction of larger trials using ivermectin for the early treatment of COVID-19. Such trials should include patients with risk factors for severe disease as well as patients with pneumonia. The potential for a mechanism of action different to direct antiviral effect also opens the door for pre-exposure prophylaxis in high-risk groups.

Author contributions

Carlos Chaccour and Aina Casellas had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conceptualization: CCh, NRR, MFA
Data curation: CCh, AC, JB
Formal analysis: AC, CCh, FH, VS
Funding acquisition: CCh, NRR
Investigation: CCh, AB, IP, AFM, PRC, MAR, MRM, CJI, FC, MG, EL, JCG, JRY, JLD, GR, BS, MFA
Methodology: CCh, AC, FC, CD, GM, FH, GR, BS, MFA
Supervision: CCh, BS, GR, MFA
Writing - original draft: CCh, AC
Writing - review & editing: all authors contributed, reviewed and approved the last draft.

Funding

Idipharma SL (Noain, Spain) contributed with in kind placebo tablets. This study was supported by ISGlobal and the University of Navarra. CCh, PRC, MAR, FH and NRR received salary support from Unitaid through the BOHEMIA grant to ISGlobal. ISGlobal acknowledges support from the Spanish Ministry of Science and Innovation through the "Centro de Excelencia Severo Ochoa 2019–2023"; Program (CEX2018–000,806-S), and support from the Generalitat de Catalunya through the CERCA Program.

Data availability

Upon publication, all data supporting the results will be archived in a public repository accessible at http://diposit.ub.edu/dspace/handle/2445/101776

Declaration of Competing Interest

JLDP reports speaker fees from Pfizer and MSD as well as research grants from Novartis, outside the scope of the submitted work. No other competing interests were disclosed

Acknowledgments

We thank the patients who participated in this study and the nursing staff of the emergency room and technicians of the microbiology lab of the Clínica and the Biobank, Universidad de Navarra for their dedication to this study.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.eclinm.2020.100720.

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DOI 10.1186/s12936-017-1801-4

FOI 26-3132 - Document 5
Malaria Journal

REVIEW

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Ivermectin to reduce malaria transmission I. Pharmacokinetic and pharmacodynamic considerations regarding efficacy and safety

Carlos Chaccour[1,2,3*], Felix Hammann[4] and N. Regina Rabinovich[1,5]

Abstract

Ivermectin is an endectocide that has been used broadly in single dose community campaigns for the control of onchocerciasis and lymphatic filariasis for more than 30 years. There is now interest in the potential use of ivermectin regimens to reduce malaria transmission, envisaged as community-wide campaigns tailored to transmission patterns and as complement of the local vector control programme. The development of new ivermectin regimens or other novel endectocides will require integrated development of the drug in the context of traditional entomological tools and endpoints. This document examines the main pharmacokinetic and pharmacodynamic parameters of the medicine and their potential influence on its vector control efficacy and safety at population level. This information could be valuable for trial design and clinical development into regulatory and policy pathways.

Keywords: Ivermectin, Endectocide, Pharmacokinetics, *Anopheles*

Background

Vector control has been a fundamental pillar for the remarkable achievements in malaria control 2000–2015 [1]. Residual transmission [2, 3] and insecticide resistance [4] are some of the challenges faced for sustaining the gains of vector control. Innovation is required to reach the ambitious goals proposed by the Global Technical Strategy for Malaria 2016–2030 [5].

Ivermectin is a mixture of two semi-synthetic analogs of the fermentation products of *Sterptomyces avermitilis*. It belongs to the macrocyclic lactone complex; its chemical structure has been reviewed elsewhere [6]. Ivermectin is an anti-parasitic medicine approved for the treatment and control of human onchocerciasis, lymphatic filariasis (LF), strongyloidiasis [7] and scabies [8]. It is also an endectocide, a drug capable of killing arthropods that feed on a treated individual, including *Anopheles* mosquitoes. This property makes mass drug administration (MDA) with ivermectin a potential tool to reduce malaria transmission [9, 10]. Such an intervention has the potential to reach malaria vectors that feed on the temporal and spatial gaps left by core vector control interventions (long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS).

This paper reviews the pharmacokinetic and pharmacodynamic properties of ivermectin that can affect the efficacy and safety of MDA campaigns for malaria transmission reduction.

Essential pharmacology

Mechanism of action

Ivermectin blocks synaptic transmission in invertebrates by binding to glutamate-gated chlorine channels in nerve and muscle, leading to hyperpolarization, paralysis and death of the invertebrate, including mosquitoes. These channels are part of the Cys-loop family of ligand-gated ion channels and ivermectin has consequently been shown to have additional effects on other members, for

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FOI 26-3132 - Document 5

instance the gamma-aminobutyric acid (GABA), histamine, and pH-sensitive chloride channels [7, 11].

In mammals, ivermectin acts as an allosteric agonist of $GABA_A$ receptor, another member of the Cys-loop family of ligand-gated ion channels. These receptors are located on neurons in many central nervous system regions (incl. the cerebral cortex, the limbic system, and thalamus) and increase chloride conductance, resulting in hyperpolarization and less formation of action potentials [12]. In vertebrates, GABA is a major inhibitory transmitter. The net effect of $GABA_A$ receptor stimulation is central nervous depression, which defines the syndrome of ivermectin toxicity in vertebrates.

The glutamate-gated chlorine channel in *Anopheles gambiae*

The glutamate-gated chlorine channel (GluCl) from *Anopheles gambiae* has recently been characterized [11]. These channels are predominantly expressed in some of the organs involved in motor and sensory systems, which explains the paralytic and other effects of the drug on mosquitoes, even at sub-lethal concentrations.

In *An. gambiae*, the gene for the GluCl can be expressed in four isoforms, but only one is insensitive to ivermectin. Little is known about the expression of these channels in wild mosquito populations. Mosquito resistance to ivermectin has not been reported, but theoretically selective over-expression of the ivermectin-insensitive isoform could develop as a resistance mechanism. However, the reduced fertility seen in mosquitoes taking sub-lethal doses of the drug [13, 14] could help delay this possibility.

Pharmacokinetics

The following discussion refers to pharmacokinetics of ivermectin in humans. Veterinary references are explicitly mentioned as such.

Absorption

Ivermectin is readily absorbed after oral administration. The absorption half-life ranges from 0.5 to 2.5 h [15, 16]. There are appreciable differences in systemic bioavailability (F) depending on mode of administration and disease state; ethanol based liquid formulations have up to twice the availability of solid formulations (AUC ratio 1.08–2.29) [15]. Infestations with worms such as *Strongyloides* can lead to paralytic ileus and severely impaired absorption of ivermectin. This has led to several patients requiring treatment with parenteral veterinary formulations [17, 18]. Time since last meal does not seem to influence ivermectin's bioavailability, although this is still subject to debate [19, 20].

Ivermectin is subject to presystemic metabolism and efflux in the gut. Intestinal cytochrome P_{450} 3A4 (CYP 3A4) can degrade ivermectin [21], and the active efflux pump P-glycoprotein (P-gp, MDR1, ABCB1), located luminally on intestinal enterocytes, transports absorbed ivermectin from the enterocyte back into the lumen [22]. Drugs or xenobiotics can induce or inhibit the activity of these mechanisms [23], as can pharmacogenetics differences, most notably in P-gp expression [24, 25].

As a lipophilic and comparatively heavy compound, ivermectin is thought to be subject to enterohepatic circulation (EHC) [16]. This is further supported by ivermectin being a substrate for important biliary efflux pumps (P-gp, and Breast cancer resistance protein (BCRP, ABCG2) [26]. Presence of EHC can increase the total exposure of a compound in that it can be absorbed multiple times, with high peak after initial administration and subsequent peaks after the compound has been excreted into bile and then reabsorbed again in the small intestine. At low doses, the peak concentration (Cmax) is proportional to the dose, but this proportionality is lost with doses equal or higher than 150 mcg/kg [27]. After a single oral dose of 150 mcg/kg, the peak is around 40 ng/ml [7, 15, 28]. The reported time needed to reach Cmax (Tmax) varies but is generally accepted to be approximately 4 h [28].

Figure 1 represents the PK curve observed by Elkassabi [28] in Sudanese patients. The relationship between plasma concentration and the mortality of mosquitoes feeding on treated individuals is reviewed in the efficacy section below.

Distribution

Ivermectin is highly lipophilic, shows a great degree of protein binding (>90%), and distributes widely in the body with a volume of distribution (V_d) of 3.1–3.5 l/kg. Owing to its lipophilicity, ivermectin partitions to adipose tissue, which increases V_d and leads to accumulation with prolonged elimination, as drug distributes back to plasma from fatty tissue [16, 29]. This can explain the different pharmacokinetic pattern seen in women and volunteers with higher body mass index. Protein binding becomes important in populations with high prevalence of malnutrition: there, lower plasma protein levels (especially hypoalbuminemia) will result in higher free concentrations of ivermectin and, subsequently, more drug effect and toxicity.

Distribution to the brain is hindered by the blood–brain-barrier. Specifically, this is mediated by ivermectin's size, which is not conducive to passive diffusion, and the presence of efflux pumps, for which ivermectin is a substrate. The primary efflux pump is the P-gp (of which ivermectin is also an inhibitor), although BCRP can also transport ivermectin [22, 26]. The blood–brain-barrier therefore restricts ivermectin's access to its toxicity target

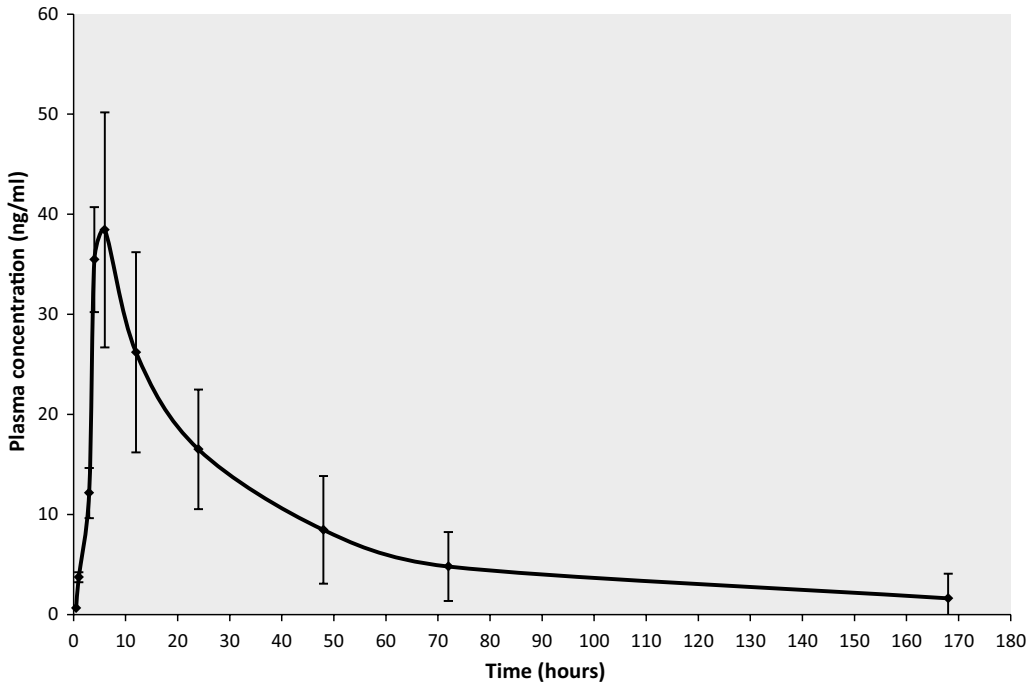


Fig. 1 The PK curve of ivermectin. Plasma concentration of ivermectin in 10 Sudanese patients infected with onchocerciasis after a single oral 150 mcg/kg dose (Data from Elkassabi [28])

in mammals, the central nervous $GABA_A$-receptor and forms the basis for ivermectin's good tolerability. P-gp expression at birth is quite low and reaches adult levels only after 6 months. This plays a large role in the susceptibility to central nervous effects from opioids [30] and possibly also for other P-gp substrates such as ivermectin. No controlled trials of ivermectin pharmacokinetics and safety have been performed in neonates and infants. In rats, however, ivermectin significantly increased postnatal mortality, presumably through exposure from maternal milk [31].

Metabolism and elimination

The plasma half-life is approximately 18 h [7]. Ivermectin is metabolized by the CYP3A4 in gut and liver [32]. The hepatic cytochrome P_{450} system at birth has 30–50% of the activity of adults [33, 34]. By consequence, failure to adjust to weight but also for decreased hepatic clearance could theoretically lead to higher than expected ivermectin exposure and toxicity in neonates and infants. Less than 1% of ivermectin is excreted unchanged in the urine (i.e. renal insufficiency will have little impact on pharmacokinetics), with most of the drug being eliminated through bile and faeces.

Ivermectin's metabolites are present at very low concentration, which makes isolation and structural characterization challenging. Authors have resorted to first identify metabolites in vitro by means of liver microsomes before attempting an in vivo characterization [27, 35]. The correlation of both systems is good in several species tested. Following this methodology, three polar metabolites: 24-hydroxymethyl-H_2B_{1a}, 24-hydroxymethyl-H_2B_{1a}-Monosaccharide and 24-hydroxymethyl-H_2B_{1b} account for up to 50% of all metabolites in liver tissue of cattle, rat and sheep in the first 14 days after dosing [27, 35]. In swine, more than two-thirds of liver residues are composed of 3″-*O*-desmethyl-H_2B_{1a} and 3″-*O*-desmethyl- H_2B_{1b} [27, 35].

In humans, studies with radio-labelled ivermectin show that peak plasma concentration of metabolites is about twice that of the parent drug and occurs later, at 7 h (vs four for parent drug) [36]. Plasma metabolites are less polar than the parent drug and could be fatty acid ester conjugates of the monosaccharides or aglycone of the parent drug [36]. The major metabolites excreted are 3″-*O*-desmethyl-H_2B_{1a} and 3″-*O*-desmethyl-H_2B_{1a}-Monosaccharide in urine and faeces respectively [36]. The plasma half-life of metabolites is about 72 h, fourfold that of the parent drug. If these metabolites have mosquitocidal activity, this could explain recent findings of a "post-ivermectin" effect in which mosquitoes feeding on treated volunteers long after the parent drug is no more identifiable in plasma still show an increased mortality [37, 38].

FOI 26-3132 - Document 5

Ivermectin is metabolized by CYP3A4 [32] but in vitro studies suggest it does not significantly inhibit its metabolizing activity or that of CYP2D6, CYP2C9, CYP1A2, and CYP2E1, all involved in its metabolism to a lower extent [7]. There is, however, a theoretical possibility of interaction with CYP3A4 inhibitors (such as protease inhibitors) or inducers such as rifampicin.

Ivermectin is both a substrate and a potent inducer of the P-gp. P-gp plays a role in the transportation of ivermectin to the intestinal lumen and in preventing its crossing of the blood-brain barrier [39]. P-gp inhibitors (such as antifungal azoles) can increase ivermectin plasma levels in animals [40, 41]. Post-marketing reports of increased International Normalized Ratio (INR) have been rarely reported when ivermectin was co-administered with warfarin [7].

The drug–drug interactions of ivermectin with artemisinin-based combination therapy (ACT) have not been well explored. Co-administration with artemether–lumefantrine was well tolerated in a small study in Burkina Faso [42], data on its safety in combination with dihydroartemisinin-piperaquine will be available from 141 participants in the IVERMAL trial [43] and further evidence on its safety in combination with dihydroartemisinin-piperaquine and primaquine will be available from the IMSEA trial [37].

Assessing the efficacy of ivermectin to kill mosquitoes

Efficacy is defined as the killing effect of ingestion of ivermectin by mosquitoes via blood (either through a direct-skin blood meal or through in vitro/membrane feeding methods). The evidence supporting this lethal effect has been reviewed extensively [9, 10, 44] and will not be re-visited here (see Additional file 1 for all studies). However, studies to assess the efficacy of ivermectin in reducing the survival of mosquitoes are not standardized. A typical approach is to allow a sample of vectors to feed on blood containing the drug or on a treated subject. Resulting mortality is assessed at intervals and reported in different formats.

The concept of lethal concentration 50 (LC_{50})

The LC_{50} is a commonly seen metric of ivermectin killing effect on mosquitoes [45, 46]; it is the concentration of ivermectin in the imbibed solution or blood meal that kills 50% of the mosquitoes during a defined period of observation. It is a measure of efficacy similar to the minimum inhibitory concentration used in bacteriology and it should not be misconstrued that the goal is to kill only 50% of the feeding mosquitoes. The LC_{50} will vary according to the time point chosen for the mortality assessment. At a given drug concentration and mosquito species, the 3-day LC_{50} will be higher than the 9-day LC_{50}, i.e. less drug is needed to kill 50% of the mosquitoes in 9 days, due to the addition of naturally occurring deaths. An alternative approach would be to determine the time to median mortality at any given concentration, but this has not been commonly used.

The feeding method used to determine the LC_{50} could also influence the measurement outcome. Ivermectin is highly lipophilic, it is found in higher concentrations in dermal and adipose tissue than venous plasma [29]. It is hypothesized that the resulting concentration gradient between the adipose tissue and the capillary blood may increase the drug concentration in the capillaries when compared to venous blood. This may be relevant as mosquitoes imbibe blood from subdermal capillaries and thus may ingest higher concentrations of ivermectin than would be predicted from the drug concentration in venous samples, i.e. mosquitoes feeding directly on the skin of a volunteer may have higher mortality than mosquitoes feeding on the blood of the same volunteer in a membrane-feeding device. Evidence is being generated with aim to clarify confounding of mortality measures by route of administration to mosquitoes, and perhaps other factors.

Pharmacokinetic considerations regarding efficacy

The efficacy of ivermectin to reduce transmission is expected to be a function mainly of its lethality to the vector population. Additional benefit will be obtained from a change in the age structure of the mosquito population: in areas of ivermectin MDA, there is increasing mortality of older biting females, this skews the mosquito population towards younger (less infectious) ages and reduces transmission beyond the initially seen lethality for up to 3 weeks [10, 47]. Additionally, older mosquitoes seem to be more susceptible to ivermectin that their younger counterparts [48].

Along these lines, the sublethal effects of the drug on mosquito fertility and flying capacity [13, 14, 45] will contribute to the overall effect (see "Other effects of ivermectin" below). Both lethality and sublethal effects will be closely related to drug concentration in reached in the blood of treated individuals *and* to the time this blood concentration is sustained.

All the concepts defined here refer to the mortality of mosquitoes feeding on a single treated person.

The mortality of mosquitoes caused by ivermectin has a dose–response gradient and is limited by the theoretical LC_{100}

The higher the concentration, the higher the mortality of mosquitoes feeding on those individuals at that time will be [42], this finding has been supported by modelling [49]. This increase in lethality will be limited by the

theoretical LC_{100} (the concentration killing 100% of biting mosquitoes, this is a theoretical concentration difficult to reach in nature). Any blood concentration above this threshold will not contribute to additional mortality (one cannot kill more than 100% of the biting mosquitoes). Figure 2 illustrates this concept.

Ivermectin's impact on mosquito mortality is directly related to the time there is a lethal concentration in the blood

The longer the drug remains in the blood, the more mosquitoes it will kill or disable. Any increase in duration of mosquitocidal concentrations is expected to contribute to additional mortality. Modelling shows that the time the drug remains in blood above mosquito-killing levels is the parameter that drives impact on transmission [50].

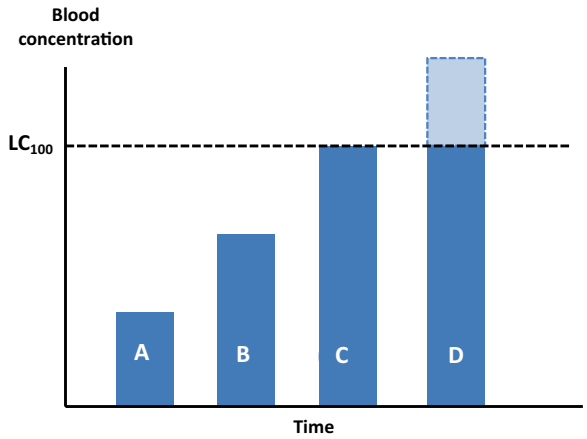


Fig. 2 The effect of increasing concentrations of ivermectin on its efficacy. *Columns A*, *B* and *C* are expected to have increasing efficacy. The area above the LC_{100} in *column D* will not contribute to further mosquito mortality. In the absence of a long elimination tail, the efficacy of the dose of *column C* and that of *column D* will be equal. *Columns* are used for illustration. LC_{100}: lethal concentration 100

The lethal effect is heterogenic

The lethality observed in any mosquito population feeding on a treated individual after a single oral dose will not be uniform. It will vary according to the plasma levels at the time of biting in close relationship with the PK of the drug. Figure 3 illustrates this concept. The total effect will be the sum of the proportions dying at different time points.

The lethal effect could be a function of the area under the curve

The blood concentration and the time the drug remains in the blood can be represented by the area under the curve (AUC). Because the blood concentration above the theoretical LC_{100} cannot add to lethality, the efficacy can be expected to be a function of the AUC that is below the LC_{100}. The AUC below LC_{100} will vary according to the magnitude of the single dose given, the number of doses, the administration route, the absorption and distribution rates of the drug as well as its metabolism and elimination. Secondary release from adipose tissue after accumulation could also play a role.

Conceptually, the ideal ivermectin dose would maximize the time drug level is near the LC100 without wasting drug going beyond the level at which most mosquitoes are killed

Given the heterogeneity of mosquito lethality in time, in the (theoretical) presence of a constant biting rate, a "peaked"

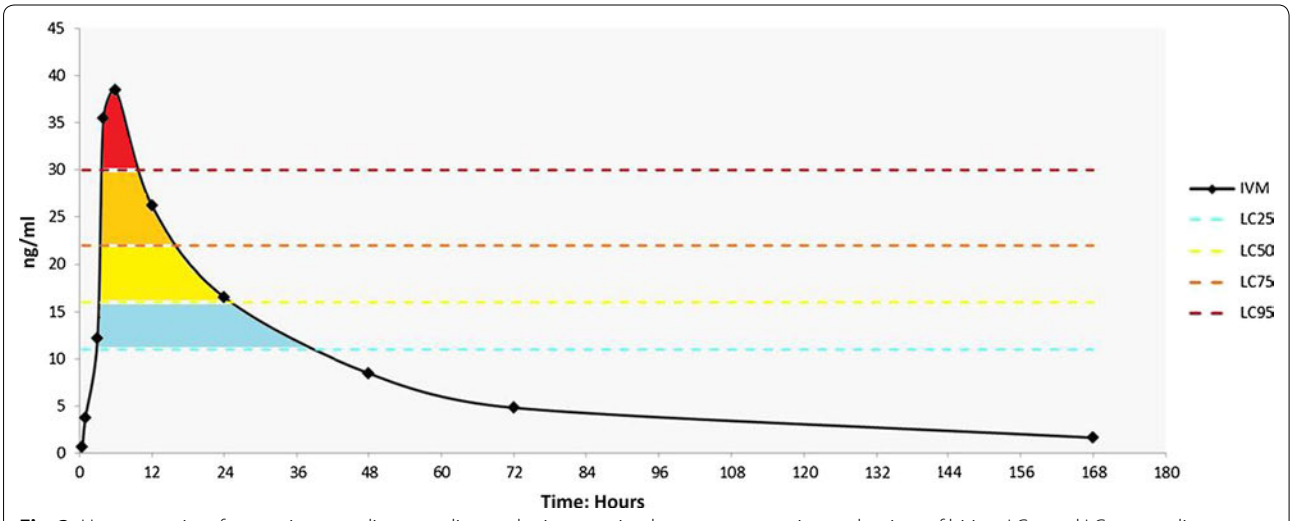


Fig. 3 Heterogeneity of mosquito mortality according to the ivermectin plasma concentration at the time of biting. LC_{50} and LC_{25} according to Kobylinski et al. [46]. The LC_{95} and LC_{75} shown have not been determined and are shown for illustration purposes. *LC* lethal concentration

curve with a Cmax close to the LC100, but with a narrow base, can have the same efficacy of a wider curve, even if the Cmax is lower. Figure 4a illustrates this concept. Ivermectin MDA could be however tailored to make the Cmax coincide with the peak biting activity of the local vectors [11].

Following this rationale and considering the point illustrated in Fig. 2, a large dose yielding a "peaked" curve with Cmax high above the LC_{100} could be less efficacious than a dosing scheme yielding the same area under the curve without surpassing the LC_{100}. This is because the AUC above the LC_{100} will not directly contribute to the efficacy. This is illustrated in Fig. 4b.

Time above lethality target

Modelling can help generate a robust hypothesis on the mosquito lethality target at a population level. This will be a function of the individual dose per body weight. The time above lethality target is related to the area under the curve but takes into consideration the susceptibility of the local mosquitoes. It can be expressed in time as a "mosquitocidal window". Figure 5 illustrates how the susceptibility of the local vector can influence this variable.

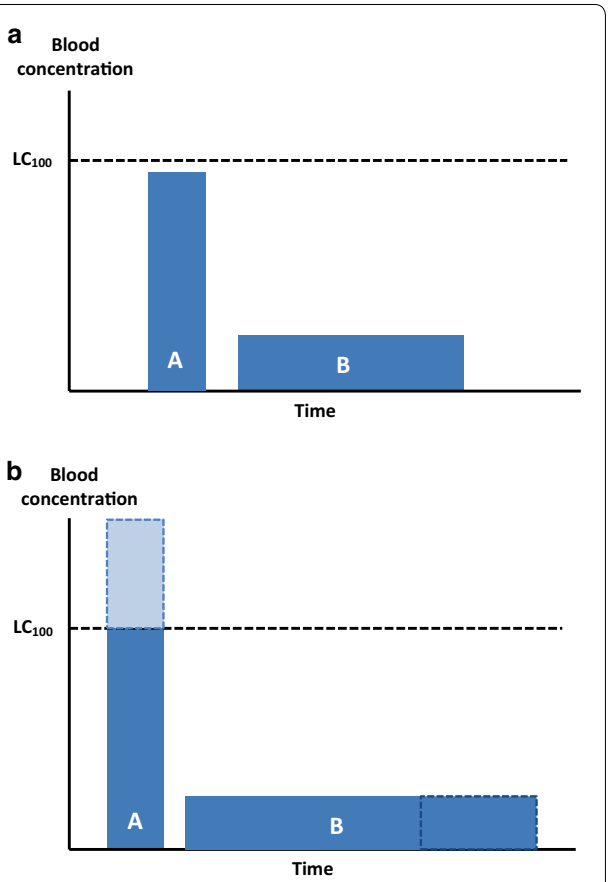


Fig. 4 AUC of different dosing schemes and their potential relationship with efficacy. **a** The area of both *columns* is the same ($A = B$), hence, in the presence of constant biting rate, the total number of mosquitoes killed by A and B might me similar, even if B does not reach the same Cmax. **b** If the theoretical LC_{100} is surpassed (*light blue area*), the drug consumed to reach such plasma levels is partly wasted because it does not contribute to efficacy and may in turn increase the possibility of side effects. *Columns* are used only for illustration. LC_{100} lethal concentration 100

Dose–response curves

The slope of the curve will represent the logarithmic increase in the AUC below LC_{100} needed to kill a higher proportion of mosquitoes. Although recent data suggests the relationship between plasma concentration and mosquito mortality is linear at the individual level [42], at the population level the relationship AUC-efficacy is unlikely to be so. Figure 6 illustrates this concept.

Options to increase the efficacy of ivermectin

Higher doses per body weight, multiple dose regimens, or slow-release formulations are all theoretical ways to increase the AUC and hence the efficacy. The duration of sublethal concentrations can play an important role in general efficacy as vectors imbibing sublethal concentrations can have a higher mortality rate due to impaired motility or temporary paralysis (knock-down). Epidemiological and PK modelling can be used to plan the doses and regimens to be tested in field trials, but care must be taken to reflect the potential importance of this additional effects.

Higher doses (increasing the Cmax)

Using higher doses per body weight will result in larger AUC driven by a higher Cmax (Fig. 7). This will result in longer time above lethal concentrations because the slope of elimination will remain the same. This is the most straight-forward method because it could be implemented using the current oral formulation at a single encounter. The main challenges with this approach include the safety of a higher Cmax that could increase toxicity while and partial drug waste due to a portion of the AUC above the theoretical LC_{100}. Acceptability in areas where previous lower ivermectin doses have been used must be part of integrated community engagement, also needed for the understanding of direct and indirect benefits that can be expected from this approach.

Multi-dose regimens

A multiple-dose regimen would result in a series of peak concentrations that could have cumulative effect depending on the frequency of the doses (Fig. 8). The effect of every dose would also be limited by the theoretical LC_{100} plateau. The main limitations of this approach are compliance and the logistics of multiple rounds of MDA. Additionally, the valleys caused by the intermittent dosing may result in "vulnerable windows" because levels

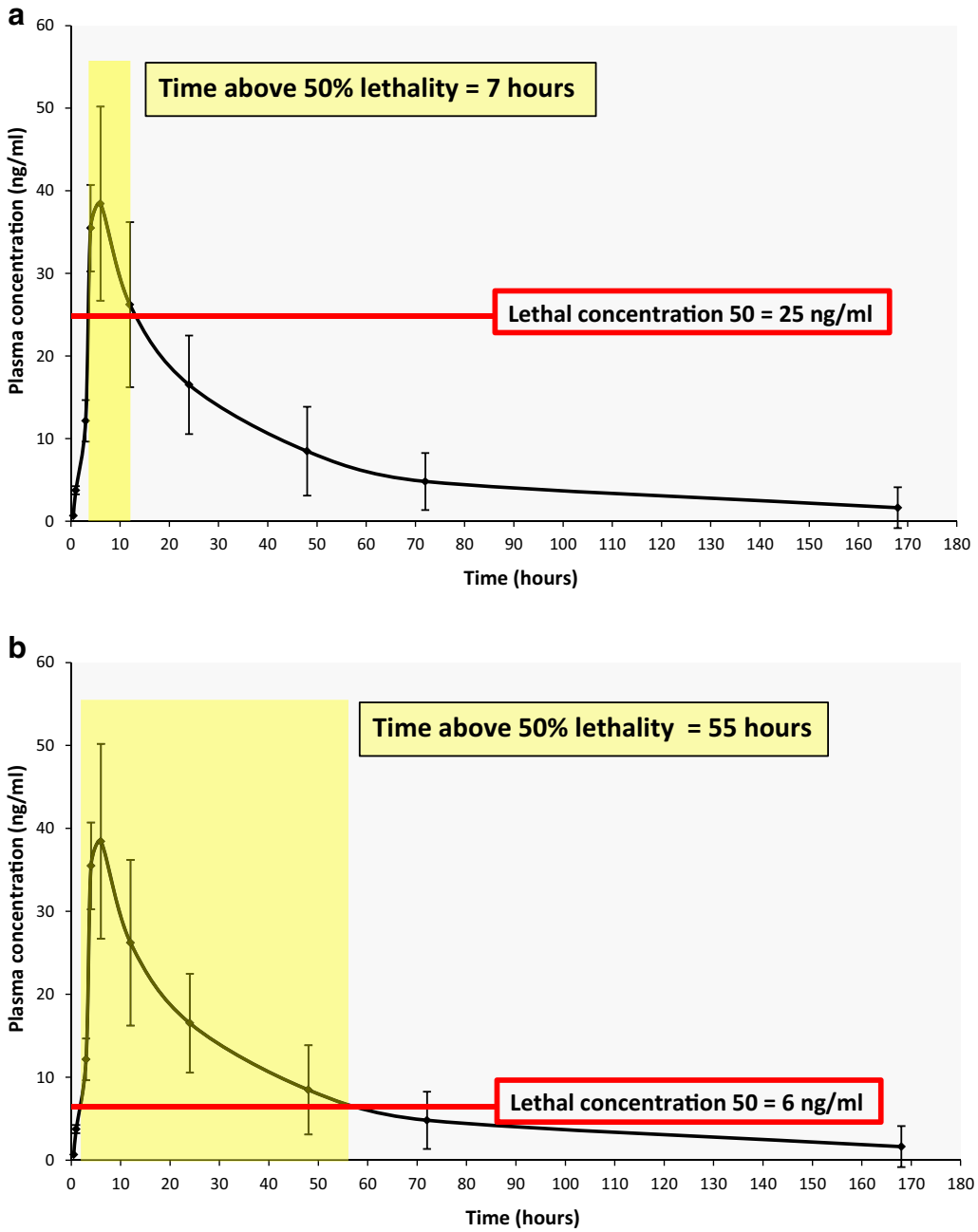


Fig. 5 The time above lethality and the "mosquitocidal window". An illustration of how the lethality target selected and the susceptibility of the local vectors can affect the efficacy of ivermectin to reduce malaria transmission. The *curves* represent the PK of ivermectin after a single oral 150 mcg/kg dose according to Elkassaby [28]. In both *panels*, the lethality target is the LC_{50}. The vectors of **a** are less susceptible and higher concentrations are required to kill 50% of them, the time above lethality target is 7 h. The vectors of **b** are much more susceptible, this increases the time above lethality eightfold. *LC* lethal concentration

might be in the insufficient dose range, decreasing efficacy. Preliminary data from a recent cluster-randomized trial showed a 20% reduction in clinical incidence of malaria in children under five by active case detection in areas where a 200 mcg/kg dose was given to all eligible population every 3 weeks for six doses [51].

Slow-release formulations

A long-lasting, slow-release formulation [52, 53] would have effect on the Cmax depending on the release rate, which, if controlled, could theoretically improve the efficacy/safety ratio (Fig. 9). The main issue with this approach is investment in R&D and the need to reassess

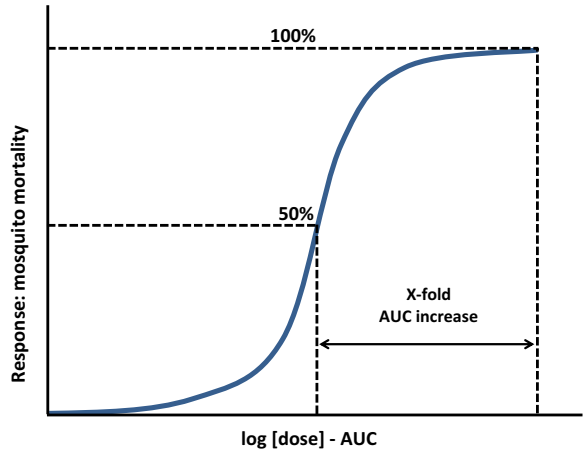


Fig. 6 Logarithmic ivermectin dose–response curve as mosquitocidal. In the case of ivermectin, the dose will be a function of the AUC and the response a function of the total mosquito mortality plus the sublethal effects. *AUC* area under the curve

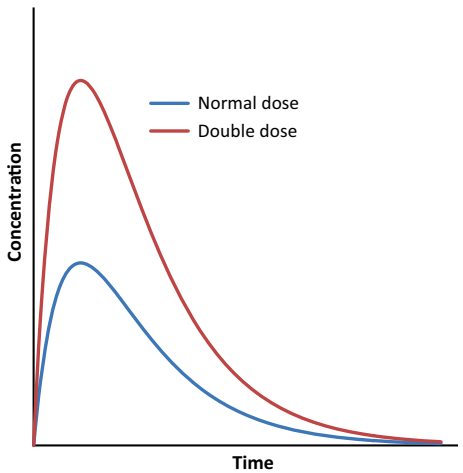


Fig. 7 Increasing efficacy using higher doses. Using a higher dose-per-body weight in a single encounter increases the AUC by increasing the Cmax. *AUC* area under the curve

the efficacy of the new formulation on the treatment of neglected tropical diseases.

Other options

One alternative to increase the AUC is to prolong the half-life of the drug (alter the elimination slope), which could theoretically be achieved with CYP3A4 and/or P-gp inhibitors. Doing so, however, may unreasonably increase the risk of toxicities and drug interactions, especially with antiretrovirals. The addition of a rather specific CYP3A4 inhibitor with no P-gp inhibition, such as voriconazole [54], would be an interesting approach as this would increase bioavailability of ivermectin while not impairing P-gp's vital function at the blood–brain barrier.

Other effects of ivermectin

In addition to the direct killing effect of ivermectin, there several sub-lethal effects that can increase the net impact of the drug on malaria transmission:

Effect on vector fertility

Several studies report diminished fertility of *Anopheles* mosquitoes after an ivermectin-loaded blood meal containing sub-lethal concentrations [13, 14]. Reduced hatching of the laid eggs has also been observed. Of note, this effect may delay but not completely avoid the appearance of ivermectin-resistant mosquitoes.

Effects on vector behaviour

Knock down, lesser flight performance and reduced tendency to bite have all been reported after taking sublethal ivermectin concentrations in a blood meal [45, 55]. These effects measured in the laboratory might contribute to greater mosquito mortality in the field.

Effects on the parasite

In the mosquito, ivermectin might inhibit plasmodium sporogony [46, 56] and could have an effect on liver schizonts as seen in vitro [57, 58] and confirmed in mouse model [57], these findings require further evaluation.

Key knowledge gaps regarding efficacy

Methods

- Lack of standardized protocols for the assessment of ivermectin's efficacy.
- Lack of correlation between the mortality observed in mosquitoes taking ivermectin through membrane vs those taking it via skin-feeding. Validating membrane feeds as a reliable, predictive assay compared to direct skin-feeding would facilitate evaluation of different approaches.

LC_{50}

The main gap is dearth of data on The LC_{50} determined via human direct skin-feeding. Results should be obtained for different species and strains in different sites, especially for known outdoor-biting species or main vectors of areas targeted for elimination.

Time above lethality

Determine the blood concentration that should be achieved with an ivermectin-based tool and how long should it be sustained in order to have a measurable impact on transmission.

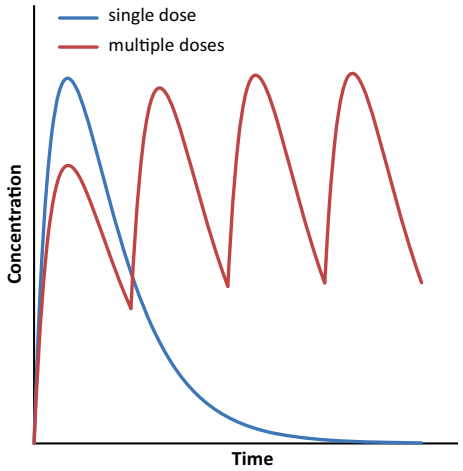


Fig. 8 Increasing efficacy by means of multiple dosing. Using a series of multiple doses can increase the AUC while avoiding reaching plasma concentrations where the efficacy/safety ratio is lower

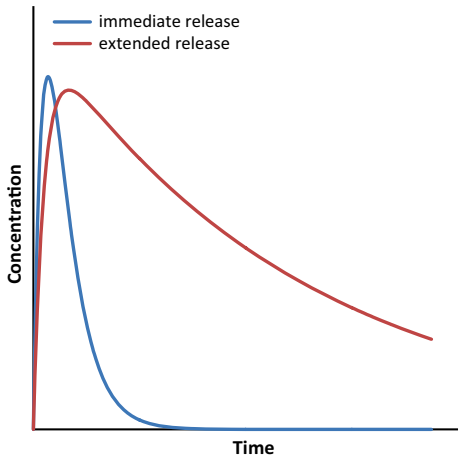


Fig. 9 Increasing efficacy with a slow-release formulation. A long lasting formulation would increase AUC by prolonging time above lethality, without significant increase in the Cmax and theoretically improving the efficacy/safety ratio. *AUC* area under the curve

Other effects

Assess whether ivermectin, by having a mechanism of action different from all public health insecticides available today, could help reduce the risk of insecticide resistance that is not CYP-mediated. Also the influence of ivermectin´s effects on mosquito fertility in potentially delaying the appearance of ivermectin-resistant mosquitoes.

PK considerations regarding safety

Ivermectin has been licensed for human use for almost 30 years, and its safety has been assessed in over 70 trials. More than 2.7 billion 150–200 mcg/kg single doses have been distributed by the Mectizan Donation programme [59]. Since community use of ivermectin implies that drug will be given to at risk and infected individuals, all for indirect benefit of lowering malaria disease rates, the safety profile and the risk–benefit assessment will be critical. For use as a vector control measure, it is the combination of the blood levels and the duration of these levels that will need to have an acceptable safety profile. Both parameters are directly related to drug dosage and regimen required to reach impact. Regarding safety, the adverse effect rate is also expected to be a function of the cumulative dose. Host factors such as illnesses and co-administered drugs must also be taken into account.

Therapeutic index

Drugs developed for elimination endeavours and MDA administration must have a high therapeutic index [60]. The therapeutic index is a measure of the range of doses that elicit a therapeutic response without unacceptable adverse effects [61]. Using ivermectin for vector control, this relationship will be between the mosquito mortality (as a proxy for efficacy) and the AUC (as a proxy for dose per body weight and number of doses). Figure 10 illustrates this concept.

As an example, Guzzo et al. administered 1.404–2.000 mcg/kg in a single dose to 16 healthy volunteers in the US (>tenfold the usual 150-200 mcg/kg single dose for onchocerciasis) and did not report a greater adverse effect rate than in controls [62]. These findings suggest the therapeutic index of ivermectin for onchocerciasis control is at least higher than 10. For malaria control the therapeutic index will be lower as the dose needed will be higher. The findings of Guzzo et al. provide a good safety reference at 3.200 mcg/kg in a week (see Table 1). The Centers for Disease Control and Prevention recommends doses of up to 1.400 mcg/kg within a month for the treatment of severe crusted scabies [63].

The efficacy/safety ratio

A particular AUC will elicit a specific efficacy/adverse effects ratio. This ratio is expected to increase exponentially with the AUC; in theory, once the LC_{100} has been reached, this ratio can only increase at the expense of the time above lethality. Using the therapeutic index concept described above, after a certain cumulative dose, toxicity will start to increase and the efficacy/adverse effects ratio will be reduced (Fig. 11). Increasing the dose per body weight given in a single encounter is a possible strategy to increase the efficacy. This strategy, however, must be carefully evaluated since the AUC above the LC_{100} will minimally contribute to efficacy and may in turn increase the risk of toxicity.

FOI 26-3132 - Document 5

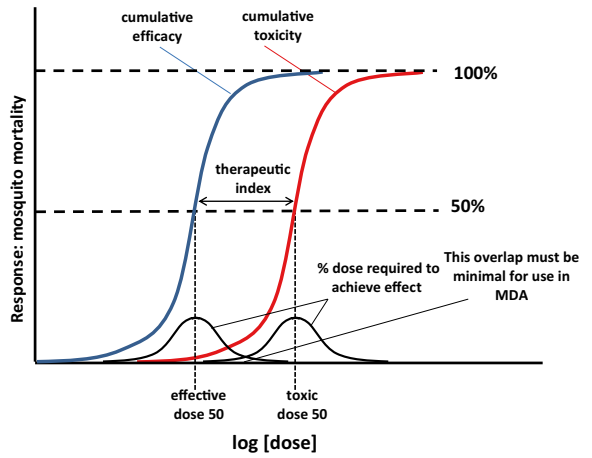


Fig. 10 Therapeutic index. *AUC* area under the curve, *MDA* mass drug administration (Adapted from Golan et al. [61])

Safety profile of ivermectin in community campaigns–implications for malaria

Since the creation of the Mectizan Donation Programme in 1988, more than 2.7 billion doses of ivermectin have been distributed for the control of onchocerciasis and lymphatic filariasis in Africa, Latin America, and Asia [59]. Exclusion criteria are: children under 15 kg, pregnant women, nursing mothers in the 1st week after delivery, the severely ill and those with known hypersensitivity to the drug [64]. Coverage target is normally between 65 and 80% of the whole population [65].

Safety of ivermectin in onchocerciasis MDA campaigns

In onchocerciasis-infected patients, adverse events (AE) to ivermectin are usually mild, transient, associated with intensity of microfilarial infection and primarily characterized as mild Mazzoti-type reactions to dying microfilaria [66]. These effects wane in subsequent administrations [67]. No significant association has been found between ivermectin plasma levels and AE recorded [68]. A recent Cochrane review of ivermectin for river blindness shows that side effects are rarely reported [69]. Outside *Loa loa* endemic areas (see below), the drug is remarkably safe.

Loa-associated encephalopathy

Loa loa is a parasitic infection that is broadly present in geographies overlapping the onchocerciasis/LF programmes. While causing limited direct disease, administration of ivermectin to individuals infested with *Loa loa* can result in encephalopathy in 0.01–0.11% of the treated population [70], if the *Loa loa* burden is high (>30.000 parasites/ml), the odds ratio can be above 1000 [70]. The syndrome includes confusion, lethargy and coma. The pathophysiology behind this syndrome is not clear, but rapid killing of *Loa* microfilariae or even defects in transmembrane efflux pumps may play a role [70, 71]. At a population level, high levels of microfilaraemia are seen in 1% of the population in areas with an overall *Loa loa* prevalence greater than 20%. This threshold was used by the Mectizan Expert Committee and the Technical Consultative Committee to define the preventive strategies recommended for ivermectin distribution in *Onchocerca* and *Loa* co-endemic areas [72]. Nonetheless, the risk of this severe adverse event excludes parts of *Loa*-endemic central Africa from ivermectin MDA campaigns for the elimination of onchocerciasis; this includes areas of Angola, Cameroon, Central African Republic, Chad, Congo, Democratic Republic of the Congo, Equatorial Guinea, Ethiopia, Gabon, Nigeria and South Sudan [73].

Several tools and strategies are emerging in response to the limitations caused by *Loa* which creates a near term window of opportunity for malaria: New diagnostic tools (loascope) allow for quantitative population screening in real time [74] and novel biomarkers could also predict burden at individual level [75]. This test and (not) treat strategy may offer a programmatic approach to addressing the *Loa* barrier to ivermectin treatment. This strategy could reduce the *Loa* burden at population level, lowering the risk of parasite-burden-related adverse reactions. Finally, single-administration of drug combos [76] can offer a rapid pathway to LF elimination, this treatment also reduces *Loa loa* burden and thus risks from ivermectin for any indication (including malaria).

Safety of ivermectin at doses higher or more frequent than approved for NTDs

A single ivermectin dose of 150–200 mcg/kg results in a too short-lasting mosquito-killing effect to be applicable for malaria impact. Therefore, for this indication, higher doses and/or multi-dose regimens than those currently used for onchocerciasis will be needed. A range of dosages are already recommended for different indications. The FDA-approved ivermectin dose for strongyloidiasis MDA is 150 mcg/kg (every 12 months), although the possibility of quarterly use in individual patients is also included in the label [7]. The French authorities recommend up to 400 mcg/kg for the control of lymphatic filariasis in selected areas [77]. For severe crusted scabies, up to seven 200 mcg/kg doses within a month in combination with topical treatment and keratolytics are recommended in the US [63] and Australia [78]. The possibility of using more than 3 doses for the treatment of moderate to severe crusted scabies cases is included in the Australian label [8].

Very few studies, at varying doses and frequencies, have evaluated the safety of ivermectin regimens at doses

Table 1 The safety of ivermectin at doses higher or more frequent than current approval

Reference	Population	Highest single dose	Maximum frequency	Maximum number of doses	Maximum total dose (period)	Adverse events
Awadzi et al. [100]	75 males with moderate to heavy *Onchocerca* infection	800 mcg/kg	Single	Single	800 mcg/kg (once)	No difference with controls taking the 150 mcg/kg dose
Awadzi et al. [101]	100 adult males infected with *Onchocerca*	800 mcg/kg	Days 1 and 4	2	1.600 mcg/kg (4 days)	No difference with controls taking the 150 mcg/kg dose
Guzzo et al. [62]	68 male and female healthy, adult volunteers	2.000 mcg/kg	Days 1, 4 and 7	3	3.273 mcg/kg (1 week)	No difference with controls
Kamgno et al. [67]	657 adult males infected with *Onchocerca*	800 mcg/kg	3-monthly	13	8.950 mcg/kg (3 years)	The high dose group reported a statistically higher rate of transitory mild and subjective visual side effects (No structural explanation). For all other AE comparable rates reported in all groups
Smit et al. [43]	141 adults with uncomplicated malaria	600 mcg/kg	Days 1, 2 and 3	3	1.800 mcg/kg (3 days)	Pending publication of results

The maximum total dose is the cumulative dose received by a participant during a given period which is given in brackets

AE adverse events

FOI 26-3132 - Document 5

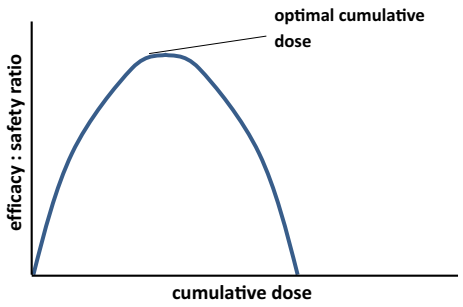


Fig. 11 The efficacy/safety ratio. The relationship between the efficacy: adverse effect ratio and the cumulative dose

above 400 mcg/kg for control of NTDs (Table 1). Pharmacokinetic modelling suggests that a regime consisting of a daily dose of 600 mcg/kg for 3 days has the potential to sustain ivermectin concentrations lethal to *Anopheles* mosquitoes for at least 1 week [43]. This is the basis of the recently finished IVERMAL trial in Kenya [43].

The skewing of the age structure of the mosquito population for around 3 weeks after a single round of MDA for onchocerciasis (150 mcg/kg) could also support reduction in transmission of malaria. This has been used as the basis for the RIMDAMAL trial [79] which consisted on six rounds of ivermectin MDA 3 weeks apart each. Preliminary data from this cluster-randomized trial shows no significant adverse events with this those [51].

Safety of ivermectin during pregnancy and lactation

Pre-clinical studies in pregnant mice, rats and rabbits show teratogenicity at doses toxic to the mother (400 mcg/kg, 5.000 mcg/kg and 3.000 mcg/kg during pregnancy days 6–18 respectively) [7, 80]. Ivermectin can produce delayed development and increase pup mortality in rats at maternal doses of 1600 mcg/kg [80]. It is estimated that in *Onchocerca*-endemic areas, up to 50% of pregnant women in the first trimester are systematically inadvertently treated with ivermectin during MDA campaigns [81].

Five studies have specifically evaluated the effects of inadvertent ivermectin treatment during pregnancy (four case–control studies and one clinical trial). The results are presented in Table 2. The studies encompass a total of 839 women treated during pregnancy, including 442 women treated in the first trimester. No difference with controls is reported regarding pregnancy outcome, newborn health status or early child development. There is however no systematic database of inadvertent exposure during pregnancy to date. Based on these results, the proscription of ivermectin treatment during pregnancy was lifted for areas where women are *at high risk of blindness*. The decision to include pregnant women, however, is left at the discretion of the program directors [82].

Low ivermectin levels are found in human breast milk after a single oral dose of 150–250 mcg/kg in healthy women with a peak at 1 h post-ingestion of 18.5 ng/ml [80, 83]. It remains detectable in human milk at very low levels (<1 ng/ml) for up to 14 days after a single dose [80]. Only nursing mothers in the 1st week after delivery are systematically excluded during MDA campaigns [64, 82].

A systematic review of the evidence regarding safety in pregnancy is needed. This is important because at population level the effectiveness of any ivermectin-based strategy will be determined by the population coverage achieved [84]. If the safety of the expected higher or more frequent doses needed for malaria is not established in pregnancy, excluding women in reproductive age is

Table 2 Five studies assessing the safety of ivermectin during pregnancy compared with community based controls

Reference	Country	Number of pregnant women	Number inadvertently treated	In the first trimester	Pregnancy outcome	Child mortality	Child development
Pacque et al. [81]	Liberia	939	200	171 (85%)	No difference with controls	No difference with controls	No difference with controls (follow-up 2 years)
Doumbo et al. [102]	Mali	461	82	Not stated	Data not readily assessable		
Chippaux et al. [103]	Cameroon	511	110	(93) 85%	No difference with controls	No difference with controls	No difference with controls (follow-up 1 year)
Gyapong et al. [104]	Ghana	343	50	(50) 100%	No difference with controls	No difference with controls	No difference with controls (follow-up not stated)
Ndyomugyenyi et al. [105]	Uganda	834	397[a]	All in 2nd trimester	No difference with controls	No difference with controls	Not included

[a] Clinical trial

FOI 26-3132 - Document 5

likely to reduce the efficacy of the intervention to reduce malaria transmission.

Safety of ivermectin in infants and children

Ivermectin is licensed for the treatment of children weighing more than 15 kg [7, 8]. In MDA campaigns a height of 90 cm is used as a proxy for 15 kg. A preclinical toxicology study in 24 neonatal (7–13 days old) rhesus monkeys showed no adverse reaction after 2 weeks of daily doses of up to 100 mcg/kg [80]. An additional study in eight immature rhesus monkeys (13–21 months old), receiving doses of up to 1.200 mcg/kg for 14–16 days showed no treatment related findings; three animals presented increased serum transaminases which was attributed to an infectious origin. In humans, there are only anecdotal case reports [85, 86] and small case series [87] of its use off-label in infants less than 15 kg.

Similarly to pregnancy, the importance of a clear guidance regarding use in children weighing less than 15 kg is directly related to ivermectin MDA efficacy. At population level, coverage will be directly proportional to efficacy [84]. Importantly, the impact of including small children on the overall efficacy of ivermectin MDA for malaria will be related to the mosquito biting rate as well as risk of transmission in this particular population.

In areas of high transmission, where most of disease burden occurs in children under 5 years, this age group is expected to receive the largest proportional benefit of ivermectin MDA to reduce malaria transmission. In the context of the RIMDAMAL trial, the main outcome measure was malaria incidence in children under 5 years of age, where most of these children did not receive ivermectin [51]. Conducting dose-ranging studies in children will allow for increased population coverage of an ivermectin-based vector control intervention.

Safety of ivermectin in high-risk groups

There is no renal or hepatic ivermectin dose defined [7]. Renal dose adjustments would not appear necessary for a drug for which less than 1% is excreted unchanged in urine. It is conceivable that active metabolites exist that are eliminated renally. There is little information available on the safety of ivermectin in patients over 65 years of age. A report of excess deaths (several causes) among 47 residents of a nursing home following ivermectin MDA for scabies (single dose 150–200 mcg/kg) [88] raised heated debate [89–92]. Ivermectin was licensed in Australia for the treatment of scabies in 2013 [93]. The elderly tend to have less adipose tissue and thus lower volumes of distribution for lipophilic drugs, such as ivermectin, which will result in higher plasma concentrations. They are also more prone to hypoalbuminaemia due to malnutrition, potentially yielding higher free concentrations of ivermectin. Lastly, hepatic function (and with that: capacity for detoxification) decreases with age.

There is no evident biological basis for concern on potential cardio-toxicity. Dukuly et al. [94] prospectively followed 32 men (mean age 61 years), including 20 with baseline EKG abnormalities and found no significant changes after ivermectin treatment.

HIV-infected individuals are not excluded from treatment based on their serological status [64]. Potential drug–drug interactions with anti retrovirals or TB drugs must be particularly taken into account when treating this special population (see drug interactions below).

Concerns about the theoretical risk of using ivermectin in patients with epilepsy have been resolved [82, 95].

Environmental concerns about ivermectin

There are three ways in which ivermectin can enter the environment: excretion from treated humans or animals, from disposal of pharmaceutical waste, or from emissions from manufacturing sites [80]. Haley et al. showed ivermectin undergoes rapid degradation in light and soil [80, 96]. This, combined with tight binding to soil and sediment prevents environmental accumulation and minimizes its potential impact on non-target organisms [80, 96]. Veterinary ivermectin formulations affect the dung fauna and there was initial concern that it may delay dung degradation [97]. More recent studies have suggest this is not the case [98].

Key knowledge gaps regarding safety

- The safety profile of ivermectin when used at higher doses, or with longer exposure treatment schemes.
- The safety of the proposed dose/schemes in populations likely to affect coverage if excluded i.e. potentially pregnant women and children under 15 kg.
- New strategies to assess and prevent the *Loa*-related adverse effects.
- The safety of ivermectin in combination with anti-malarials and other drugs commonly used in endemic areas such as antiretrovirals, TB drugs and other antihelmintics.

Conclusions

Ivermectin MDA has the potential to reduce malaria transmission by increasing the mortality of malaria vectors biting treated individuals, particularly those only partially affected by LLINs and IRS due to behavioural or physiological resistance. A thorough understanding of the pharmacological properties of ivermectin is pivotal to design studies aiming at providing evidence for a policy recommendation.

Ivermectin is safe in MDA campaigns at the current dose approved for onchocerciasis and LF 150–200 mcg/

FOI 26-3132 - Document 5

kg administered not more than four times a year. If used for malaria control, the dose and administration scheme will change. The efficacy of an ivermectin-based tool will be directly related to coverage; hence all population groups should be represented in the safety data collections, including women in reproductive age, children and the elderly. Additional preclinical safety studies might be needed to include these groups in clinical trials. The appropriate best time to include susceptible groups would be after the dose, formulation and administration scheme have been defined.

Additional file

Additional file 1. Studies assessing the efficacy of ivermectin to kill mosquitoes taking a loaded blood meal.

Abbreviations

AUC: area under the curve; AE: adverse events; BBB: blood–brain-barrier; BCRP: breast cancer resistance protein; GABA: gamma-aminobutyric acid; GluCl: glutamate-gated chlorine channel; HIV: human immunodeficiency virus; INR: International Normalized Ratio; IRS: indoor residual spraying; LC_{50}: lethal concentration 50; LF: lymphatic filariasis; MDA: mass drug administration; NTDs: neglected tropical diseases; P-gp: p-glycoprotein; PK: pharmacokinetic; TB: tuberculosis; V_d: volume of distribution.

Authors' contributions

CCh and RR prepared the first draft. All authors contributed to and reviewed the last version of the manuscript. All authors read and approved the final manuscript.

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Acknowledgements

Carlos Chaccour is supported by a Ramón Areces fellowship. This work was partly supported by the PIUNA scheme from the University of Navarra. ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya.

Competing interests

The authors declare that they have no competing interests.

Declarations

This publication is based on the background document prepared by the authors for the WHO Technical Consultation on "Preferred product characteristics of ivermectin for malaria transmission control", held in Geneva 30 March–1 April 2016. The main conclusions of this consultation were presented to the Malaria Policy Advisory Committee of WHO, and can be found at the Malaria Policy Advisory Committee website [99]. This manuscript solely represents the author´s perspective after discussion with advisory groups and scientific collaborators.

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Received: 9 January 2017 Accepted: 6 April 2017
Published online: 24 April 2017

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FOI 26-3132 - Document 5

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International Journal of Sciences
FOI 26-3132 - Document 5
Research Article Volume 9 – September 2020 (09)

Effectiveness of Ivermectin in SARS-CoV-2/COVID-19 Patients

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Abstract: Background:The first case of Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were diagnosed in Wuhan, China in 2019. In the first half of 2020 this disease has already converted into a global pandemic. Objectives: To assess the efficacy of Ivermectin in mild cases of COVID-19 patients on the basis of predefined assessment criteria. Study Settings: Fatima Memorial Hospital, Lahore Study Design: Open label randomized control trial. Duration of Study: From 1st May, 2020 to 30th June, 2020.Patients & Methods: Sample size and technique: Sample size was 50 patients; 25 patients were kept in control group and 25 patients were kept in experimental group. Results: There were total 50 patients, divided into two groups case and control group.The mean age of the participants was 40.60 ± 17 and out of those 31 (62%) were male and 19 (38%) were females. Cough was observed more in case group (p= 0.049). Fever, myalgias and dyspnea were the commonest symptoms in both the groups (p= 1.000). Diarrhea and vomiting were more common in control group (p=0.0001, p= 0.042 respectively). On follow up at day 7, patients were stratified as asymptomatic and symptomatic. Amongst the case group, out of 25 patients, 16(64%) patients were asymptomatic and rest of the 9(36%) patients was asymptomatic. In control group, out of 25 patients, 15(60%) patients were asymptomatic and rest of the 10(40%) patients was asymptomatic. p-value (0.500). Statistically there was no significant difference between case group who were given ivermectin along with symptomatic treatment and control group who were only given symptomatic treatment without ivermectin, being asymptomatic on day 7 at follow up. p-value (0.500)

Keywords: Corona Virus, Covid-19, SARS-COv-2, Antiviral, Ivermectin, Pandemic

Introduction:

The first case of Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were diagnosed in Wuhan, China in 2019. In the first half of 2020 this disease has already converted into a global pandemic, having different forms of presentation in different patients.

Exponential rise in its cases along with associated mortality has shaken the world.[1]

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a single-stranded ribonucleic acid (RNA) virus that causes a severe acute respiratory syndrome. The virus was originally called SARS-CoV-2 named officially by World Health Organization as COVID-19 and a global health emergency. The first known case of infection was recorded in early December 2019 and subsequently spread to various continents, including Europe and the United States[2, 3]Generally, 75 percent of patients recover without any notable complication however 25 percent can experience associated complications leading to intensive care unit transfer and even mortality.[4]

Ivermectin has different effects to treat variety of diseases and it has proven antimicrobial, antiviral, and anti-cancer properties and considered as a wonder drug. It is highly effective against many microorganisms including some viruses.[5] Many studies revealed its antiviral effects on RNA viruses such as Zika, dengue, yellow fever, West Nile, Hendra, Newcastle, Venezuelan equine encephalitis, chikungunya, Semliki Forest, Sindbis, Avian influenza A, Porcine Reproductive and Respiratory Syndrome, Human immunodeficiency virus(HIV) type 1, and severe acute respiratory syndrome coronavirus SARS-CoV-2. Fon other hand there are few studies showing antiviral effects of ivermectin against DNA viruses such as Equine herpes type 1, BK polyomavirus, pseudorabies, porcine circovirus 2, and bovine herpesvirus 1.[5] Ivermectin plays a vital role in several biological mechanisms, therefore it could emerge as a potential candidate in the treatment of a wide range of viruses including COVID-19 as well as other types of positive-sense single-stranded RNA viruses. In vivo studies of animal models revealed a broad range of antiviral effects of ivermectin, however, more clinical trials are necessary as it's the need of the hour to do so, as we can validate the potential efficacy of ivermectin in clinical settings.

The recent report by Caly et al.[6] describing the antiviral potential of ivermectin against the severe

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Published at: http://www.ijsciences.com/pub/issue/2020-09/
DOI: 10.18483/ijSci.2378; Online ISSN: 2305-3925; Print ISSN: 2410-4477

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FOI 26-3132 - Document 5

acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in vitro arrive to the agenda of potential candidates for COVID-19 treatment.[6]

Currently, there is not any Food and drug administration (FDA) approved specific effective antiviral treatment available for COVID-19.Although most of the COVID-19 patients have mild or moderate courses, up to 5%–10% can have severe, potentially life threatening course, there is an urgent need for development of effective & safe drugs to deal with this novel virus. Optimized supportive care remains the mainstay of therapy. There are more than 300 clinical trials going on, various antiviral and immunomodulating agents are in different stages of evaluation for COVID-19 in those trials and results/ primary end points of few trials will be published in the next couple of months. Despite the urgent need to find an effective and safe antiviral treatment for COVID-19 through randomized controlled studies, certain agents are being used all over the world based on either in-vitro or conclusive evidence or observational studies.[7]

The clinical efficacy and utility of ivermectin in SARS-CoV-2-infected patients is unpredictable at this stage, as we are dealing with a completely novel virus.

There is an urgent need for investigation of the mortality causes and development of novel therapeutic options for severe COVID-19.

Operational definition:
COVID-19 infection:
Patients were labeled to have mild disease due to acute respiratory syndrome coronavirus 2 (SARS-CoV-2; previously named as 2019-nCoV) proven via RT-real time COVID-19 polymerase chain reaction (PCR) test.

Methodology:
Study Design:
It was a open label randomized control trial where participants were allocated using predefined protocol who met the inclusion criteria of study after signing a written & informed consent either by the patient or the person legally authorized to sign.

Study Settings:
Study was conducted in Fatima Memorial Hospital, Lahore among patients reporting to COVID-19 clinics and medical outpatient department OPD.

Sample size and technique:
our sample size was 50 patients; 25 patients were kept in control group and 25 patients were in experimental group. Patients were allocated randomly to the groups by computer generated number. Sampling technique was convenient sampling as per the inclusion and exclusion criteria. It was an open label randomized control trial which was done on COVID-19 patients proven by RT- PCR fulfilling the criteria (mild severity).

Duration of study: Starting after IRB approval, 1st May, 2020 to 30th June 2020

Inclusion Criteria:
- All patients diagnosed with COVID-19 infection with positive reverse transcriptase RT-PCR test, who were willing to participate in this study
- Patients having age of 18-75 years
- Patients of both genders male and female
- Patients who had mild symptoms of Corona virus disease and RT- PCR positive for SARS-Cov-2
- Ability to take oral medication and were willing to adhere to the drug intake regimen

Exclusion Criteria:
- Known severe allergic reactions to Ivermectin
- Pregnancy or breastfeeding
- Severe symptoms likely attributed to Cytokine Release Storm
- Malignant diseases
- Chronic kidney disease
- Cirrhosis liver with Child class B or C

Data Collection and analysis Procedure: All patients having COVID-19 proven by RT-PCR reporting to outpatient department OPD and clinics at Fatima memorial Hospital were studied. Patients were allocated randomly to the groups by computer generated number. Data regarding Epidemiological, demographic, clinical, and treatment was collected and analyzed. Outcomes were noted. All the data entered on a structured Performa (Annex-I)

Response of the patients after Ivermectin prescription and response of the patients without Ivermectin prescription was recorded. Response was recorded on the basis of clinical parameters (Fever, Cough, sore throat, Headache, Shortness of breath, lethargy, and fatigue. Any side effects noted after prescription of Ivermectin was recorded.

Ivermectin Prescribing protocol:
Patients were prescribed Ivermectin 12mg stat and then 12 mg after 12 hours and 12mg after 24 hours and we looked at the response at day 7 on follow up in terms of improvement of symptoms like (Fever, Cough, sore throat, Headache, Shortness of breath, lethargy, and fatigue.

Data was entered and analyzed using SPSS 25.0. Frequency and percentages were calculated for the qualitative variables like gender, comorbidity, symptoms, response to treatment. Quantitative variables of the study like age were expressed as Mean ± SD

Ethical Considerations:
Patients were included in the study after written and informed consent. Institutional review board IRB approval was taken from the ethical committee.

Results:
Total of fifty patients were included in our study, the mean age of the participants was 40.60 ± 17 and out of those 31 (62%) were male and 19 (38%) were females. Patients were divided in two groups, case and control. Case group was offered the symptomatic treatment along with Ivermectin and control group was given only symptomatic treatment without Ivermectin. Control group particpants' were older than the case group statistically but there is no difference between the average ages of both groups (p= 0.582). There was no association between the gender and case group (p= 0.561). Cough was observed more in case group (p= 0.049). Fever, myalgias and dyspnea were the commonest symptoms in both the groups (p= 1.000). Diarrhea and vomiting were more common in control group (p=0.0001, p= 0.042 respectively). Regarding loss of taste and and anosmia, were more seen in case group (p=0.0009) Table-1

Table: 1 -Age & Gender distribution of the participants & Comparative analysis of groups at presentation

Variables	Group		Total	p-value
	Case	**Control**		
Age	40.60 ±17	43.08 ± 14.8	41.84±15.695	0.582
Gender Male	17 (34%)	14(28%)	31(62%)	0.561
Female	8 (16%)	11(22%)	19 (38%)	
Cough	24 (48%)	18(36%)	42(84%)	0.049
Fever	25 (50 %)	24 (48 %)	49(98%)	1.000
Sore throat	20 (40%)	14(28%)	34(68%)	0.128
Headache	20(40%)	13(26%)	33(66%)	0.072
Dyspnea	25(50%)	25(50%)	50(100 %)	------
Nausea	6(12%)	10 (20%)	26(32%)	0.364
Vomiting	6(12%)	14(28 %)	20(40%)	0.042
Diarrhea	4(8%)	17(34 %)	21(42%)	0.000
Myalgia	23(46 %)	24(48%)	47(94%)	1.000
Loss of taste	15(30%)	5(10%)	20(40%)	0.009
Anosmia	15(30%)	5(10%)	20(40%)	0.009
Hypotension	2(4 %)	6(12 %)	8(16 %)	0.247

Age is presented as Mean±SDand for comparison independent sample t test is applied
All the categorical variables are presented in N (%) and Fisher's Exact Test is applied

On follow up at day 7, patients were stratified as asymptomatic and symptomatic. Amongst the case group, out of 25 patients, 16(64%) patients were asymptomatic and rest of the 9(36%) patients was asymptomatic. In control group, out of 25 patients, 15(60%) patients were asymptomatic and rest of the 10(40%) patients was asymptomatic. p-value (0.500) statistically there is no significant difference between case group who were given ivermectin along with symptomatic treatment and control group who were only given symptomatic treatment without ivermectin, being asymptomatic on day 7 at follow up. p-value (0.500) Table-2

In case group, adverse reaction of ivermectin like heartburn was seen in 8(32%) patients and reported as well.

Table-2: Comparative analysis of groups on Follow up at day 7

Group	**follow up @Day 7**		
	Asymptomatic	Symptomatic	Total
Case	16 (64%)	9(36%)	25
Control	15(60%)	10(40%)	25
Total	31 (62%)	19(38%)	50(100%)

Discussing about the comorbidities, out of all these 50 patients, there were 20(40%) patients who had diabetes mellitus, 11(22%) in case group and 9(18%) in control group. Hypertension was seen in 13(26%) patients, 7(14%) in case group and 6(12%) in control group. There were 6(12%) obese patients, 2(%4) in case group and in 4 (8%) in control group. Cardiovascular disease was observed in 4 (8%) patients and each group had 2(4%) patients. There were 15(30%) patients who were active smokers, 9 (18%) in case group and 6(12%) in control group. There was no patient with chronic lung disease in our study (Table-3)

Table: 3.Frequency of comorbidities

Comorbidity	*Total*	*Case group*	*Control group*
DM*	20 (40%)	11(22%)	9 (18%)
HTN+	13(26%)	7(14%)	6(12%)
Obesity	6(12%)	2(4%)	4(8%)
CVD$	4(8%)	2(4%)	2(4%)
Smoking	15(30%)	9(18%)	6(12%)

DM* Diabetes mellitus, HTN+ Hypertension, CVD$ Cardiovascular disease

Discussion:

In this study, Effectiveness of Iveremectin was assessed among the participants by looking at the symptoms on day 7 at follow up. To best of our knowledge, very few studies of this kind had been conducted in our region. We report that patients on presentation had fever, myalgias being the commonest symptoms found in both groups' patients presenting to our hospital.

Regarding recovery at Day 7 on follow up, case group vs control group, 16 (64%) Vs 15 (60%) and a study conducted in Bangladesh[8] revealed recovery in terms of being symptom free was seen in 55% patients which is comparable with our study results, however the study conducted in Bangladesh was a comparative study between two groups receiving two different treatment regimens, one group received ivermectin with doxycycline and other group received azithromycin with hydroxychloroquine.

A study conducted by Patel A[9] on 169 critically sick patients in early days of Covid-19 showed survival benefit in 18.3% of patients, however this study took all the critical patients of Covid-19 and in contrast to our study which was only conducted on mild cases of COVID-19 and our study revealed that after giving Ivermectin, on day 7, 64% patients were symptom free (recovery). A huge difference in recovery between our study and the study conducted by Patel A[9] can be attributed to the patients' selection in both the studies. We selected only mild disease patients with Covid-19 whereas study by Patel A[9] selected all the critically sick patients including mechanically ventilated patients.

A huge multicentre study named as ICON (Ivermectin in Coronavirus disease nineteen) conducted in Covid-19 patients in South Florida[10,11] enrolled 280 admitted patients and mean age of the participants was 59.6 ±17.9 years and our study participants mean age was 42±17 years which is comparable to some extent as we only took out patients and study by Rajter JC[10] only enrolled all the admitted patients, so elder patients got admitted more.

In the study conducted by Rajter JC[10] there were two groups, one treated with ivermectin and other group was given conventional treatment other than Ivermectin, there was statistically significant difference between two groups' mortality 15% vs 25% and also mortality was also lower in 75 patients having severe pulmonary disease treated who were given ivermectin (38.8% vs 80.7%). Ivermectin group showed less mortality during treatment of COVID-19, especially in patients who were on higher inspired oxygen or ventilatory support. In contrast our study was first of its kind in our country conducted on outpatients having mild disease, showed recovery in 64% patients.

COVID-19 pandemic has affected over 200 countries in a span of less than 4 months burdening on the entire health care systems of many countries with no FDA approved drug which can stop the viral transmission or infectivity. Most of the drugs which are being used to treat this novel virus are not so effective and the development of vaccines is under preparation and may be available on trial basis in future, therefore, there is urgent need of the day to develop and use drugs cautiously. Ivermectin which is famous and used frequently as antiparasitic drug but in recent past studies have shown that Ivermectin has antiviral activity. It is easily available in our country and highly affordable as well. Ivermectin is officially approved for use in SARS-cov patients in few countries like Peru and Bolivia.[12]

COVID-19 has hit the whole world resulting in a significant mortality as well as leaving a huge impact on every country's economy. Various treatment options are still under study. Recently ivermectin's activity against SARS-CoV-2 gives ray of hope, but off-label and compassionate use requires careful risk–benefit considerations, especially in critically ill patients. A path to consider is evaluation first of impacts on virologic outcomes in uncomplicated, low-risk patients early in the course of the disease. Well-conducted clinical trials informed by robust pharmacokinetic models should be considered to validate the impact before the use of ivermectin to treat SARS-CoV-2 is implemented.

Conclusion:

Our study didn't show statistically any significant difference between case and control group. In Ivermectin's (case group) recovery was almost equal to control group who received only conventional symptomatic treatment, so this is the need of the day that we need to conduct more randomized controlled trials across our country involving major tertiary care health care facilities with larger sample size to assess its efficacy for validating the use of Ivermectin against SARS-CoV-2. Nearly 40 clinical trials are ongoing world over for measuring the outcome of COVID-19 treatment with Ivermectin.

FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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Systematic Review and Meta-Analysis

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Comparative efficacy and safety of pharmacological interventions for severe COVID-19 patients

An updated network meta-analysis of 48 randomized controlled trials

Qinglin Cheng, PhD[a,b,*], Gang Zhao, PhD[a], Junfang Chen, BS[a], Qingjun Jia, MD[a], Zijian Fang, MD[a]

Abstract

Background: To date, there has been little agreement on what drug is the "best" drug for treating severe COVID-19 patients. This study aimed to assess the efficacy and safety of different medications available at present for severe COVID-19.

Methods: We searched databases for randomized controlled trials (RCTs) published up to February 28, 2022, with no language restrictions, of medications recommended for patients (aged 16 years or older) with severe COVID-19 infection. We extracted data on trials and patient characteristics, and the following primary outcomes: all-cause mortality (ACM), and treatment-emergent adverse events (TEAEs).

Results: We identified 4021 abstracts and of these included 48 RCTs comprising 9147 participants through database searches and other sources. For decrease in ACM, we found that ivermectin/doxycycline, C-IVIG (i.e., a hyperimmune anti-COVID-19 intravenous immunoglobulin), methylprednisolone, interferon-beta/standard-of-care (SOC), interferon-beta-1b, convalescent plasma, remdesivir, lopinavir/ritonavir, immunoglobulin gamma, high dosage sarilumab (HS), auxora, and imatinib were effective when compared with placebo or SOC group. We found that colchicine and interferon-beta/SOC were only associated with the TEAEs of severe COVID-19 patients.

Conclusion: This study suggested that ivermectin/doxycycline, C-IVIG, methylprednisolone, interferon-beta/SOC, interferon-beta-1b, convalescent plasma (CP), remdesivir, lopinavir/ritonavir, immunoglobulin gamma, HS, auxora, and imatinib were efficacious for treating severe COVID-19 patients. We found that most medications were safe in treating severe COVID-19. More large-scale RCTs are still needed to confirm the results of this study.

Abbreviations: ACM = all-cause mortality, CI = confidence interval, C-IVIG = hyperimmune anti-COVID-19 intravenous immunoglobulin, COVID-19 = coronavirus disease 2019, CP = convalescent plasma, CQ = chloroquine, DS = duration of study, HCQ = hydroxychloroquine, HS = high dosage sarilumab, IFN-β = interferon beta, IG = immunoglobulin gamma, LPV/r = lopinavir/ritonavir, LS = low dosage sarilumab, NMA = network metaanalysis, OR = odds ratio, RCTs = randomized controlled trials, RRB = risk of reported bias, SOC = standard of care, SS = sample size, SUCRA = surface under the cumulative ranking area, TEAEs = treatment-emergent adverse events, WHO = World Health Organization.

Keywords: efficacy, network meta-analysis, randomized controlled trials, safety, severe COVID-19

1. Introduction

To date, the World Health Organization has confirmed over 480 million cases.[1] The mortality in patients with COVID-19 was estimated at 1.28%.[1] For the current analyses, COVID-19 infection is the leading cause of the global burden of disease and public health, which has increased significantly since 2019, driven mainly by high morbidity, high impact, and mortality.[2]

Analysis of the data showed many pharmacologic interventions have been used to treat COVID-19 patients, which have been effective against COVID-19 infection.[3–5] However, there were many compounds that differ in efficacy and safety, and it was not clear which drug was the "best" drug for treating severe COVID-19 patients. Due to the small sample size and time-updated lagged in previous meta-analyses and studies, there has been debate about the effectiveness and safety of

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are publicly available.

Supplemental Digital Content is available for this article.

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How to cite this article: Cheng Q, Zhao G, Chen J, Jia Q, Fang Z. Comparative efficacy and safety of pharmacological interventions for severe COVID-19 patients: An updated network meta-analysis of 48 randomized controlled trials. Medicine 2022;101:41(e30998).

Received: 25 May 2022 / Received in final form: 1 September 2022 / Accepted: 6 September 2022

http://dx.doi.org/10.1097/MD.0000000000030998

drugs, with some findings contradicting each other for COVID-19 infection.[6,7]

Of primary concern was the medications on COVID-19 with all infection levels (i.e., mild, moderate and severe infection) in prior studies.[8] Here, we had a critical shortage that we lacked an analysis stratified by different infection levels in medications of COVID-19. It is well known that the disease varies in infection, which could lead to individual treatment of different infection subgroups in patients.[9]

Though the network metaanalysis (NMA) has been performed in prior reports,[7] there was little published data on the network study for the therapy of severe COVID-19 patients. Additionally, we found that there have been further randomized controlled trials (RCTs) of some other drugs for the treatment of COVID-19 through the literature search.[10] Some large-scale RCTs have been completed in the treatment of COVID-19 infection. We urgently need an updated assessment of the available evidence to support clinical decision-making.

How do we select pharmacological interventions for severe COVID-19 patients in clinical practice? In order to fill this gap, we did an updated NMA of RCTs for drug interventions of severe COVID-19 infection. The purpose of this study is to evaluate the efficacy and safety of medications based on RCTs of the available evidence for severe COVID-19 infection.

2. Materials and Methods

2.1. Protocol and registration

We performed the present NMA in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement.[11] This study was a review article and did not involve a research protocol requiring approval by a relevant institutional review board or ethics committee. Informed consent was also not applicable. We registered the protocol in the International Prospective Register of Systematic Reviews (PROSPERO) (CRD42021293879).

2.2. Search strategy and selection criteria

We conducted a systematic literature search in 8 electronic databases (PubMed, Elsevier Science Direct, Cochrane Library, Google Scholar, Springer Link, MedRxiv, China National Knowledge Infrastructure, and Wanfangdata) for RCTs published, with no language restrictions from their inception to February 28, 2022. We included the RCTs on the treatment of severe COVID-19 patients (aged 16 years or older). The appendix had full search strategies listed (Supplemental Appendix 1, http://links.lww.com/MD/H533). We extracted data on RCTs, patient and medications characteristics (Table 1).

To determine their eligibility, we reviewed the abstracts and full-texts of potentially relevant articles. We selected articles for the evaluation based on the criterion: at least one statistical analysis of the association between severe COVID-19 and medications was presented and described as an assessment for efficacy or safety.

All RCTs that measured the efficacy or safety between drug interventions and severe COVID-19 infection were considered for inclusion. We listed full inclusion and exclusion criteria in the appendix (Supplemental Appendix 2, http://links.lww.com/MD/H534). During the selection of qualified studies, we resolved any ambiguity through mutual discussion and consensus.

2.3. Data extraction

At least 2 independent investigators (JQJ, CJF, and FZJ) extracted and entered onto all data through a standardized data extraction form. The main data extracted was the assessments of efficacy and safety. We collected the following information: basic characteristics, including author name, publication year, country/countries of origin, study design, method of COVID-19 testing, patient population, age, gender, sample size, interventions, treatment medication dose, controls, control medication dose, follow-up time, and primary outcomes; Primary outcomes, including all-cause mortality (ACM) and rate of treatment-emergent adverse events (TEAEs). We also contacted the authors if they did not report the above data information in the published article. One independent investigator undertook a preliminary extraction of studies, and another investigator reviewed the extraction. Differences were discussed, and a third researcher participated (CQL) if they reached no agreement.

2.4. Quality assessment

At least 2 investigators (ZG, CQL, JQJ, CJF, and FZJ) evaluated the risk of bias for all studies. We estimated the risk of bias with the Cochrane Risk-of-Bias Tool.[12] We assessed the certainty of evidence by using the Grading of Recommendations Assessment, Development, and Evaluation approach for the NMA.[13]

2.5. Outcome measures and definitions

The severe COVID-19 infection represented patients with fever or suspected respiratory infection, plus one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO2 ≤ 93% on room air.[14,15] The primary outcomes were the ACM and TEAEs for severe COVID-19 patients, from the beginning of treatment to the end of follow-up. The definitions of ACM, TEAEs, and severe COVID-19 patients can refer to our previous study.[14]

2.6. Data analysis

2.6.1. Assessment of the transitivity assumption. Transitivity is the fundamental assumption of indirect comparisons and NMA. We investigated the distribution of potential effect modifiers to assess the transitivity assumption. Potential effect modifiers included multicenter study, duration of study (DS), blinding, crossover design, sample size (SS), industry sponsorship, and risk of reported bias (RRB).

2.6.2. Network meta-analysis. We used STATA statistical software (Version 15, Stata Corporation, and College Station, Texas, USA) to conduct NMAs. We analyzed binary variables (ACM and TEAEs) using odds ratio (OR) with 95% confidence interval (CI). The appendix reported additional details (Supplemental Appendix 3, http://links.lww.com/MD/H535). We defined statistical significance as the P value < .05.

We merged simultaneously the direct evidence and indirect evidence or different indirect evidence through an NMA. In NMA, we analyzed the effect of intervention using group-level data. A fixed-effect model (i.e., $I^2 \leq 50\%$) or a random-effect model (i.e., $I^2 > 50\%$) was used to summarize the effect sizes of NMA. We ranked the therapeutic effect for each outcome using the surface under the cumulative ranking area (SUCRA) curve and mean ranks.[16] In addition, the endpoints which lower was better would show rank 1 was the best and rank N was the worst based on figures and vice versa.

2.6.3. Assessment of heterogeneity and inconsistency. We used the node splitting method (i.e., split evidence on a specific comparison into direct and indirect evidence) to estimate the inconsistency of NMAs.[17] There was no significant inconsistency in outcomes if $P > .05$.

Table 1

Characteristics of randomized controlled trials of pharmacological interventions in severe patients with COVID-19.

Author name (reference)	Publication yr	Country/ Countries of origin	Study design	Method of COVID-19 testing	Numbers of participants	Gender*	Age (yrs)	Interventions	Treatment medication dose	Controls	Control medication dose	Follow-up time (d)	Primary outcomes
Absalón-Aguilar A et al [18]	2021	Mexico	TRPCTs	RT-PCR	116	Males (n = 76); Females (n = 40)	≥ 18	Colchicine (n = 56)	Patients received 1.5 mg of colchicine at baseline, which corresponded to the day of the patient's recruitment in the study, and, then, 0.5 mg PO BID for 10 days	Placebo (n = 60)	Patients received placebo PO at baseline, which corresponded to the day of the patient's recruitment in the study, and, then, 0.5 mg PO BID for 10 days	10	ACM; ratio of TEAEs
Ader F et al [19]	2022	Europe	MDRCTs	RT-PCR	328	Not specified	≥ 18	Remdesivir/SOC (n = 161)	Remdesivir was administered intravenously at a loading dose of 200 mg on day 1 followed by a 100 mg, 1-h infusion once-daily for a total duration of 10 days	SOC (n = 167)	Lopinavir–ritonavir (LPV/R), LPV/R and interferon beta-1a, or hydroxychloroquine	29	ACM
Ali S et al [20]	2021	Pakistan	RCTs	RT-PCR	50	Males (n = 35); Females (n = 15)	≥ 18	Hyperimmune anti-COVID-19 Intravenous Immunoglobulin (C-IVIG) (n = 40)	5% C-IVIG dosage arms (0.15, 0.20, 0.25, 0.30 g/kg)	SOC (n = 10)	Not specified	28	ACM; ratio of TEAEs
AlQahtani Met al.[21]	2021	Bahrain	RCTs	RT-PCR	40	Males (n = 32); Females (n = 8)	≥ 21	Convalescent plasma (n = 20)	The dosage of CP was 400 mL, given as 200 mL over 2 h over 2 successive days	SOC (n = 20)	Possible therapy including antiviral medications, tocilizumab and anti-bacterial medication	28	ACM; ratio of TEAEs
AlShehry Net al. [22]	2021	Saudi Arabia	RCTs	RT-PCR	164	Males (n = 137); Females (n = 27)	≥ 18	Convalescent plasma/SOC (n = 40)	300 mL (200–400 mL/treatment dose) convalescent plasma at least once, and if required, daily for up to 5 sessions	SOC (n = 124)	Not specified	30	ACM
Aman Jet al. [10]	2021	Netherlands	DRPCTs	RT-PCR	385	Males (n = 264); Females (n = 121)	≥ 18	Imatinib (n = 197)	800 mg on day 0 followed by 400 mg daily on d 1–9	Placebo (n = 188)	Not specified	28	ACM
Avendano Sola C et al [23]	2020	Spain	MRCTs	RT-PCR	81	Males (n = 44); Females (n = 37)	≥ 18	Convalescent plasma (n = 38)	Receive 1 dose (250-300 mL) of convalescent plasma	SOC (n = 43)	Not specified	97	ACM; ratio of TEAEs
Beltran-Gonzalez JLet al. [24]	2021	Mexico	DRPCTs	RT-PCR	106	Males (n = 66); Females (n = 40)	53 ± 16.9	Hydroxychloroquine (n = 33)	Hydroxychloroquine 400 mg every 12 h on the first day and subsequently, 200 mg every 12 h for 4 days	Placebo (n = 37)	Not specified	30	ACM
Beltran-Gonzalez JL et al [24]	2021	Mexico	DRPCTs	RT-PCR	106	Males (n = 66); Females (n = 40)	53 ± 16.9	Ivermectin (n = 36)	Ivermectin, 12 mg or 18 mg	Placebo (n = 37)	Not specified	30	ACM

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FOI 26-3132 - Document 5
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Table1

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Author name (reference)	Publication yr	Country/ Countries of origin	Study design	Method of COVID-19 testing	Numbers of participants	Gender*	Age (yrs)	Interventions	Treatment medication dose	Controls	Control medication dose	Follow-up time (d)	Primary outcomes
Bruen Cet al[25]	2022	USA	DRPCTs	RT-PCR	261	Males (n = 176); Females (n = 85)	≥ 18	Auxora/SOC (n = 130)	Auxora was administered by a 4-h IV infusion at 2.0 mg/kg (1.25 mL/kg) at 0-h and 1.6 mg/kg (1 mL/kg) at 24 and 48 h	Placebo/ SOC (n = 131)	Placebo was dosed at 1.25 mL/kg at 0-h and 1 mL/kg at 24 and 48 h	60	ACM
Cao Bet al.[26]	2020	China	RCTs	RT-PCR	199	Males (n = 120); Females (n = 69)	≥ 18	Lopinavir/ritonavir (n = 99)	Lopinavir–ritonavir (400 mg and 100 mg: respectively) twice a day for 14 days	SOC (n = 100)	Not specified	28	ACM; ratio of TEAEs
Cao Yet al.[27]	2020	China	RPCTs	RT-PCR	41	Males (n = 24); Females (n = 17)	≥ 18	Ruxolitinib/SOC (n = 20)	5 mg twice a day	Placebo (n = 21)	Not specified	28	ACM; ratio of TEAEs
Caricchio R et al [28]	2021	Europe and the United States	DRPCTs	RT-PCR	454	Males (n = 267); Females (n = 187)	≥ 18	Canakinumab/ SOC (n = 225)	A single intravenous infusion of canakinumab (450 mg for body weight of 40-<60 kg, 600 mg for 60-80 kg, and 750 mg for > 80 kg; n = 227)	Placebo/ SOC (n = 223)	Not specified	29	ACM; ratio of TEAEs
Cremer PCet al.[29]	2021	USA	MDRPCTs	RT-PCR	40	Males (n = 26); Females (n = 14)	≥ 18	Mavrilimumab (n = 21)	Receive mavrilimumab 6 mg/kg as a single intravenous infusion	Placebo (n = 19)	Not specified	28	ACM; ratio of TEAEs
Davoudi-Monfared Eet al.[30]	2020	Iran	RCTs	RT-PCR	81	Males (n = 44); Females (n = 37)	≥ 18	Interferon-beta-1a/SOC (n = 42)	44 mg/mL (12 million IU/mL) dose of interferon-beta-1a 3 times weekly for 2 consecutive weeks	SOC (n = 39)	HCQ (400 mg twice a day [BID] on the first day and then 200 mg BD) plus LPV/r (400 and 100 mg: respectively: BD) or atazanavir-ritonavir (300 and 100 mg: respectively: daily) for 7–10 days	28	ACM
de Alencar JCGet al. [31]	2021	Brazil	DRPCTs	RT-PCR	135	Males (n = 80); Females (n = 55)	≥ 18	N-acetylcysteine (NAC, n = 67)	N-acetylcysteine (NAC,28 mg/mL and 14 mg/mL)	Placebo (n = 68)	Dextrose 5% in water (1000 mL in total) intravenously	9	ACM
Dequin PFet al.[32]	2020	France	DRPCTs	RT-PCR	149	Males (n = 104); Females (n = 45)	≥ 18	Hydrocortisone (n = 76)	200 mg/d until day 7, 100 mg/d for 4 days and 50 mg/d for 3 days, for a total of 14 days	Placebo (n = 73)	Not specified	14	ACM
Edalatifard M et al [33]	2020	Iran	RCTs	RT-PCR	62	Males (n = 39); Females (n = 23)	≥ 18	Methylprednisolone/SOC (n = 34)	Intravenous injection: 250 mg per day for 3 days	SOC (n = 28)	HCQ sulfate: lopinavir and naproxen	48	ACM

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Author name (reference)	Publication yr	Country/ Countries of origin	Study design	Method of COVID-19 testing	Numbers of participants	Gender*	Age (yrs)	Interventions	Treatment medication dose	Controls	Control medication dose	Follow-up time (d)	Primary outcomes
Ely EWet al[34]	2022	Argentina, Brazil, Mexico, and the USA	MDRPCTs	RT-PCR	101	Males (n = 55); Females (n = 46)	≥ 18	Baricitinib/SOC (n = 51)	Baricitinib 4 mg plus SOC was crushed for nasogastric tube delivery (or given orally when feasible) and given once daily for up to 14 days or until discharge from hospital, whichever occurred first	Placebo/ SOC (n = 50)	Matched placebo plus SOC	60	ACM; ratio of TEAEs
Furtado RHMet al.[35]	2020	Brazil	MDRCTs	RT-PCR	397	Males (n = 262); Females (n = 135)	≥ 18	Azithromycin/ SOC (n = 214)	500 mg azithromycin once daily plus SOC for 10 days	SOC (n = 183)	SOC without macrolides: at the discretion of treating hysicians and according to local guidelines	29	ACM
Gharebaghi Net al.[36]	2020	Iran	DRPCTs	RT-PCR	59	Males (n = 41); Females (n = 18)	≥ 18	Immunoglobulin gamma (n = 30)	IVIg (human) flebogamma 5% DIF GRIFOLS daily for 3 consecutive days	Placebo (n = 29)	Not specified	28	ACM
Hashim HAet al.[37]	2021	Iraq	RCTs	RT-PCR	140	Males (n = 73); Females (n = 67)	16 to 86	Ivermectin/ doxycycline (n = 70)	Treated with 200ug/kg PO of Ivermectin per day for 2-3 days along with 100 mg PO doxycycline twice per day for 5-10 days	SOC (n = 70)	Not specified	28	ACM
Hernandez-Cardenas Cet al. [38]	2021	Mexico	DRPCTs	RT-PCR	214	Males (n = 161); Females (n = 53)	≥ 18	Hydroxychloro-quine (n = 106)	200 mg every 12 h, for 10 days	Placebo (n = 108)	Not specified	30	ACM; ratio of TEAEs
Krolewiecki Aet al. [39]	2021	Argentina	RCTs	RT-PCR	32	Males (n = 17); Females (n = 15)	≥ 18	Ivermectin (n = 20)	Patients in the ivermectin group received oral treatment for 5 consecutive days with either breakfast or lunch at approximately 24 h intervals. Ivermectin 6 mg ranurated tablets (IVER P, Laboratorios Elea/Phoenix, and Argentina) were used in all cases at a dose of 600 mg/kg/day based on baseline weight rounding to the lower full (6 mg) and half (3 mg) dose.	SOC (n = 12)	Not specified	30	Ratio of TEAEs

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FOI 26-3132 - Document 5
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Table1

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Author name (reference)	Publication yr	Country/ Countries of origin	Study design	Method of COVID-19 testing	Numbers of participants	Gender*	Age (yrs)	Interventions	Treatment medication dose	Controls	Control medication dose	Follow-up time (d)	Primary outcomes
Lescure FXet al.[40]	2021	Argentina, Brazil, Canada, Chile, France, Germany, Israel, Italy, Japan, Russia, and Spain	MDRPCTs	RT-PCR	416	Males (n = 261); Females (n = 155)	≥ 18	Sarilumab 200 mg (n = 159)	Intravenous sarilumab 200 mg	Placebo (n = 84)	Not specified	60	ACM; ratio of TEAEs
Lescure FXet al.[40]	2021	Argentina, Brazil, Canada, Chile, France, Germany, Israel, Italy, Japan, Russia, and Spain	MDRPCTs	RT-PCR	416	Males (n = 261); Females (n = 155)	≥ 18	Sarilumab 400 mg (n = 173)	Intravenous sarilumab 400 mg	Placebo (n = 84)	Not specified	60	ACM; ratio of TEAEs
Li Let al.[41]	2020	China	MRCTs	RT-PCR	103	Males (n = 60); Females (n = 43)	≥ 18	Convalescent plasma/SOC (n = 52)	The dose of convalescent plasma was approximately 4 to 13 mL/kg of recipient body weight.	SOC (n = 51)	Not specified	74	ACM
Libster Ret al.[42]	2021	Argentina	DRPCTs	RT-PCR	160	Males (n = 60); Females (n = 100)	≥ 65	Convalescent plasma (n = 80)	Convalescent plasma 250 mL with an IgG titer greater than 1:1000	Placebo (n = 80)	Not specified	25	ACM
Miller Jet al.[43]	2020	American	RCTs	RT-PCR	30	Males (n = 14); Females (n = 16)	≥ 18	Auxora (n = 20)	Auxora was administered on 3 consecutive days as a 4-h continuous intravenous infusion. The initial dose was 2.0mg/kg (max 250 mg): and subsequent doses were 1.6 mg/kg (max 200 mg) at 24 and 48 h	SOC (n = 10)	Not specified	30	ACM
Munch MWet al.[44]	2021	Denmark, Sweden, Switzerland and India	MDRPCTs	RT-PCR	30	Males (n = 24); Females (n = 6)	≥ 18	Hydrocortisone (n = 16)	Hydrocortisone 200mg per day for 7days or until discharge	Placebo (n = 14)	Not specified	90	ACM
Olender SAet al.[45]	2020	United States	RCTs	RT-PCR	1114	Males (n = 672); Females (n = 442)	≥ 18	Remdesivir (n = 298)	Remdesivir 200 mg on day 1: followed by remdesivir 100 mg daily on days 2–5; or SOC plus remdesivir 200 mg on day 1: followed by remdesivir 100 mg daily on days 2–10	SOC (n = 816)	Allowed to receive medications that may potentially treat COVID-19: excluding remdesivir	30	ACM

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FOI 26-3132 - Document 16

Table1

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Author name (reference)	Publication yr	Country/ Countries of origin	Study design	Method of COVID-19 testing	Numbers of participants	Gender*	Age (yrs)	Interventions	Treatment medication dose	Controls	Control medication dose	Follow-up time (d)	Primary outcomes
Patel Jet al.[46]	2021	UK	DRPCTs	RT-PCR	806	Males (n = 577); Females (n = 229)	18 to 79	Otilimab (human anti-GM-CSF monoclonal) (n = 403)	A single dose of otilimab resulted in mean Cmax of 20.2 μg/mL after dosing on Day 1 and 1.9 μg/mL on Day 7	Placebo (n = 4 03)	Not specified	28	ACM
Rahmani Het al. [47]	2020	Iran	RCTs	RT-PCR	66	Males (n = 39); Females (n = 27)	≥ 18	Interferon-beta-1b (n = 33)	Patients in the interferon group received interferon-beta-1b (250 mcg subcutaneously every other day for 2 consecutive weeks) along with the national protocol medications	SOC (n = 33)	Patients received only the national protocol medications (lopinavir/ritonavir or atazanavir/ritonavir plus hydroxychloroquine for 7–10 days	28	ACM
Rasheed AMet al. [48]	2020	Iraq	RCTs	RT-PCR	49	Not specified	≥ 18	Convalescent plasma (n = 21)	Not specified	SOC (n = 28)	Not specified	60	ACM; ratio of TEAEs
Rea-Neto Aet al.[49]	2021	Brazil	RCTs	RT-PCR	105	Males (n = 70); Females (n = 35)	≥ 18	Chloroquine (CQ)/hydroxychloroquine (HCQ) (n = 53)	CQ 450 mg BID on day 1 and 450 mg once daily from day 2 to 5 and HCQ 400 mg BID on day 1 and 400 mg once daily from day 2 to 5	SOC (n = 52)	Not specified	28	ACM
Rea-Neto A et al[49]	2021	Brazil	RCTs	RT-PCR	105	Males (n = 70); Females (n = 35)	≥ 18	Chloroquine (CQ) (n = 24)	CQ 450 mg BID on day 1 and 450 mg once daily from day 2 to 5	SOC (n = 52)	Not specified	28	ACM
Rea-Neto Aet al.[49]	2021	Brazil	RCTs	RT-PCR	105	Males (n = 70); Females (n = 35)	≥ 18	Hydroxychloroquine (HCQ) (n = 29)	HCQ 400 mg BID on day 1 and 400 mg once daily from day 2 to 5	SOC (n = 52)	Not specified	28	ACM
Rosas IOet al.[50]	2021	Europe and North America	MDRPCTs	RT-PCR	438	Males (n = 306); Females (n = 132)	≥ 18	Tocilizumab (n = 294)	8 mg per kilogram of body weight, with a maximum dose of 800 mg	Placebo (n = 144)	Not specified	28	ACM; ratio of TEAEs
Sehgal ISet al.[51]	2021	India	DRPCTs	RT-PCR	42	Males (n = 29); Females (n = 13)	≥ 18	Mycobacterium-w (n = 20)	Each dose of 0.1 mL Mw contains 0.5 × 109 heat killed Mycobacterium w, 0.9% sodium chloride, and 0.01% thimerosal (as preservative)	Placebo (n = 22)	0.9% sodium chloride, 0.01% thiomersal	28	ACM; ratio of TEAEs
Shi Let al. [52]	2021	China	DRPCTs	RT-PCR	100	Males (n = 56); Females (n = 44)	≥ 18	Human umbilical cord-derived mesenchymal stem cells (UC-MSCs) (n = 65)	UC-MSCs (4 × 107 cells per infusion)	Placebo (n = 35)	Not specified	28	Ratio of TEAEs

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FOI 26-3132 - Document 5

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Table1

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Author name (reference)	Publication yr	Country/ Countries of origin	Study design	Method of COVID-19 testing	Numbers of participants	Gender*	Age (yrs)	Interventions	Treatment medication dose	Controls	Control medication dose	Follow-up time (d)	Primary outcomes
Simonovich VAet al. [53]	2020	Argentina	RPCTs	RT-PCR	333	Males (n = 225); Females (n = 108)	≥ 18	Convalescent plasma (n = 228)	Received convalescent plasma	Placebo (n = 105)	Not specified	30	ACM; ratio of TEAEs
Sivapalasingam Set al. [54]	2021	USA	DRPCTs	RT-PCR	126	Males (n = 531); Females (n = 219)	≥ 18	Sarilumab 200 mg group (n = 50)	Intravenous sarilumab 200 mg	Placebo (n = 25)	Not specified	22	ACM; ratio of TEAEs
Sivapalasingam Set al. [54]	2021	USA	DRPCTs	RT-PCR	126	Males (n = 531); Females (n = 219)	≥ 18	Sarilumab 400 mg group (n = 51)	Intravenous sarilumab 400 mg	Placebo (n = 25)	Not specified	22	ACM; ratio of TEAEs
Solanich Xet al.[55]	2021	Spain	RCTs	RT-PCR	55	Males (n = 23); Females (n = 21)	≥ 18	Methylpredniso-lone pulses (n = 27)	methylprednisolone pulses of 120 mg/day had to be admin-istered on 3 consecutive days	SOC (n = 28)	Not specified	56	ACM
Temesgen Z et al [56]	2021	USA and Brazil	DRPCTs	RT-PCR	479	Males (n = 310); Females (n = 169)	≥ 18	Lenzilumab (n = 236)	Three doses of lenzilumab (600 mg, each) or placebo were administered 8 hours apart via a 1-hour IV infusion	Placebo (n = 243)	Not specified	28	ACM; ratio of TEAEs
Veiga VCet al.[57]	2021	Portugal	DRCTs	RT-PCR	129	Males(n = 88); Females(n = 41)	≥ 18	Tocilizumab/SOC (n = 65)	Single intravenous infusion of 8 mg/kg	SOC (n = 64)	Not specified	15	ACM
Zhang Jet al. [58]	2021	China	RCTs	RT-PCR	56	Males (n = 36); Females(n = 20)	18 to 80	High-dose intravenous vitamin C (HDIVC) (n = 27)	50 mL every 12 h for 7 days at a rate of 12 mL/hour	Placebo (n = 29)	Bacteriostatic water for injection (50 mL every 12 h for 7 days at a rate of 12 ml/hour)	28	ACM
Zhong Met al.[59]	2020	China	RPCTs	RT-PCR	17	Males (n = 13); Females (n = 4)	51 to 91	α-Lipoic acid (n = 8)	1200 mg/d, intravenous infusion	Placebo (n = 9)	Equal volume saline infusion (placebo) for 7 days	30	ACM

ACM = all-cause mortality, DRPCTs = double-blind, randomized placebo-controlled trials, COVID-19 = coronavirus disease 2019, MDRCTs = multicenter, double-blind, randomized controlled trials, MDRPCTs = multicenter, double-blind, randomized placebo-controlled trials, MRCTs = multicenter, randomized controlled trials, RCTs = randomized controlled trials, RPCTs = randomized placebo-controlled trials, RT-PCR = reverse transcription-polymerase chain reaction, SOC = standard of care, TEAEs = treatment-emergent adverse events, TRPCTs = three-blind, randomized placebo-controlled trials, UC-MSCs = human umbilical cord-derived mesenchymal stem cells.

*If the total number of gender is not equal to the number of participants, it is due to loss to follow-up from participants.

FOI 26-3132 - Document 6

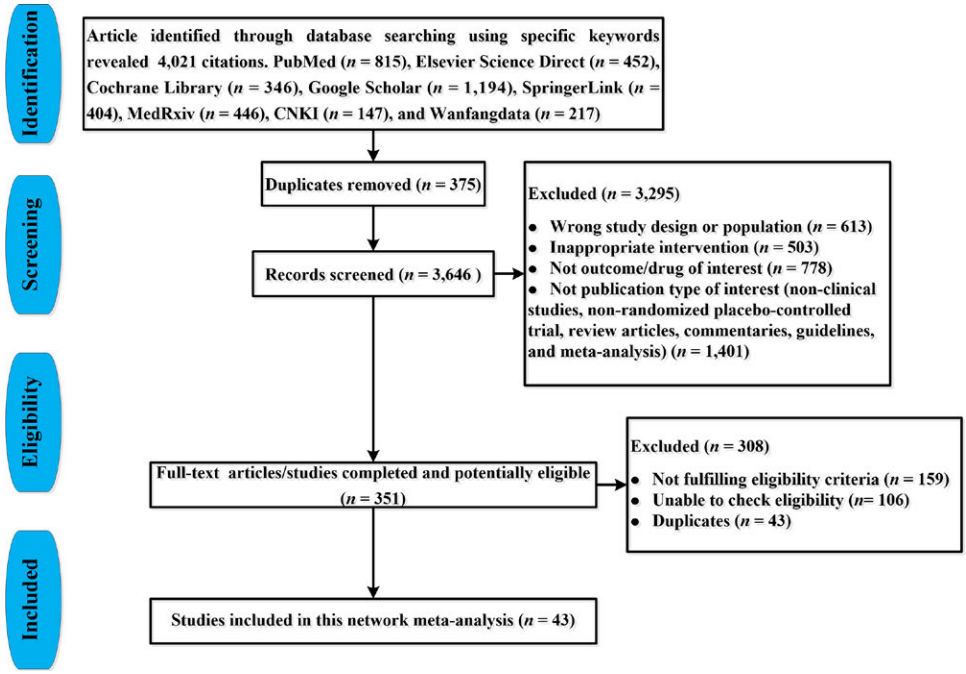


Figure 1. PRISMA flow-chart for study selection.

2.6.4. Assessment of the risk of bias. We assessed the risk of bias of included studies using the Cochrane Collaboration's Tool for Assessing Risk of Bias,[12] classifying the risk of bias as high, unclear, or low. We evaluated the small-study effect using comparisonadjusted funnel plots.[17]

2.6.5. Sensitivity analysis. The inclusion of various study designs and populations might contribute to heterogeneity and inconsistency. We used sensitivity analysis to evaluate the effect of our conclusions. We analyzed the data using the following restrictions: multicenter study, DS, blinding, crossover design, SS, industry sponsorship, and RRB.

We used the "netmeta" package in R (version 4.0.5) to duplicate the NMAs of primary outcomes.

3. Results

3.1. Description of included studies

The search identified 4021 citations, out of which 3670 were excluded for duplications, wrong study design or population (i.e., non-severe COVID-19 infection), inappropriate intervention, not outcome/drug of interest, non-clinical studies, non-RCTs, review articles, commentaries, guidelines, and meta-analysis by checking titles and abstracts. The full texts of 351 articles were obtained to check eligibility, in which we excluded 308 articles for non- fulfilling eligibility criteria, unable to check eligibility and duplications. Finally, 43 studies[10,18–59] were included in our NMA. Figure 1 shows the selection process for the included studies.

Forty-eight RCTs, including 9147 patients, were included (Fig. 1) and described in Table 1. This analysis had a mean sample size of 106 [interquartile range 58–238]. The age of subjects was greater than or equal to 16 years. The median duration of follow-up treatment was 28 days (interquartile range 27–30).

3.2. Quality appraisal

The included RCTs were of good quality (Fig. 2). Figure 2 also showed the risk of bias summary.

3.3. Network of evidence

In the network of connected RCTs (Fig. 3), the width of the lines corresponded to the number of trials included each treatment comparison. From Figure 3, we could see that the result was well connected. As shown in Figure 3A, the standard of care (SOC) was the most well-connected treatment, with chloroquine (CQ), hydroxychloroquine (HCQ), CQ/HCQ, convalescent plasma (CP), CP/SOC, remdesivir, remdesivir/SOC, lopinavir/ritonavir (LPV/r), interferon-beta-1b (IFN-β-1b), C-IVIG (i.e., a hyperimmune anti-COVID-19 intravenous immunoglobulin), ivermectin/doxycycline, IFN-β/SOC, tocilizumab, ruxolitinib/SOC, methylprednisolone, azithromycin/SOC, and auxora directly connected to it. Sarilumab such as high dosage sarilumab (HS) and low dosage sarilumab (LS), CQ/HCQ, ivermectin, canakinumab, colchicine, baricitinib, immunoglobulin gamma (IG), α-Lipoic acid, imatinib, mavrilimumab, lenzilumab, mycobacterium-w, otilimab, N-acetylcysteine, tocilizumab, high-dose intravenous vitamin C, hydrocortisone, and CP were directly connected to placebo in this network plot. Several sources of indirect evidence were available to inform comparisons between ivermectin, tocilizumab, HCQ, CP, auxora, placebo, and SOC (Fig. 3A). In Figure 3B, there was also a direct connection between SOC and C-IVIG, CP, LPV/r, ruxolitinib/SOC, ivermectin, IFN-β/SOC, and tocilizumab, or between placebo and HS, LS, mycobacterium-w, mavrilimumab, colchicine, canakinumab, baricitinib, human umbilical cord-derived mesenchymal stem cells, HCQ, lenzilumab, CP or tocilizumab. Several indirect connections were available between SOC and CP, tocilizumab, placebo (Fig. 3B).

3.4. Efficacy outcomes

Forty-one studies[10,18–38,40–51,53–59] reported ACM as outcome measurement (Supplemental Table S1, http://links.lww.com/MD/H536). We found that C-IVIG (OR 0.22, 95% CI 0.05-0.95), methylprednisolone (OR 0.27, 95% CI 0.09-0.77), IFN-β/SOC (OR 0.30, 95% CI 0.11-0.83), CP (OR 0.49, 95% CI 0.26-0.94), remdesivir (OR 0.58, 95% CI 0.37-0.93), and HS (OR 0.45, 95% CI 0.20-0.99) were associated with the decrease of ACM when compared with the SOC group (Fig. 4). Unfortunately, there was

FOI 26-3132 - Document 5

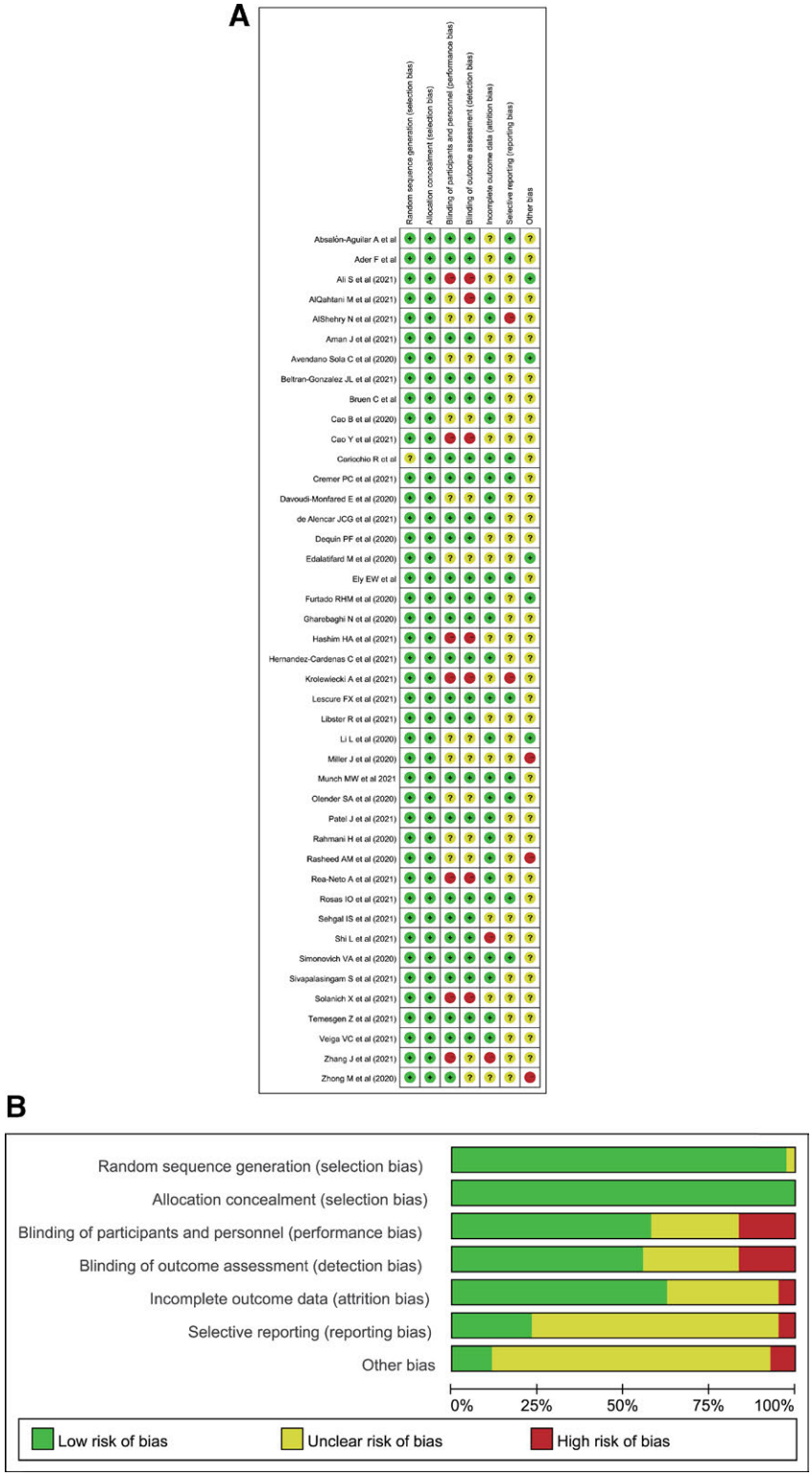


Figure 2. The quality of the included randomized controlled trials. (A) Risk of bias summary (Note: The yellow circle with question mark represents "unclear risk of bias", the red one with minus sign represents "high risk of bias" and the green one with plus sign represents "low risk of bias"). (B) Risk of bias graph.

no significant difference in other 27 medications (e.g., ivermectin/doxycycline, IFN-β-1b, LPV/r, IG, LPV/r, and imatinib) or placebo for ACM when compared with SOC.

For a decrease in ACM, ivermectin/doxycycline (OR 0.06, 95% CI 0.00-0.88), C-IVIG (OR 0.12, 95% CI 0.02-0.61), methylprednisolone (OR 0.15, 95% CI 0.04-0.52), IG (OR 0.27, 95% CI 0.08-0.85), IFN-β/SOC (OR 0.17, 95% CI 0.05-0.56), IFN-β-1b (OR 0.16, 95% CI 0.03-0.98), auxora (OR 0.37, 95% CI 0.18-0.78), imatinib (OR 0.49, 95% CI 0.25-0.96), CP (OR 0.27, 95% CI 0.11-0.64), remdesivir (OR 0.32, 95% CI 0.14-0.74), and LPV/r (OR 0.32, 95% CI 0.12-0.85) were effective when compared with the placebo group (Fig. 4). We did not identify that there was a difference between placebo and other 22 medications (e.g., ruxolitinib/SOC, ivermectin, tocilizumab,

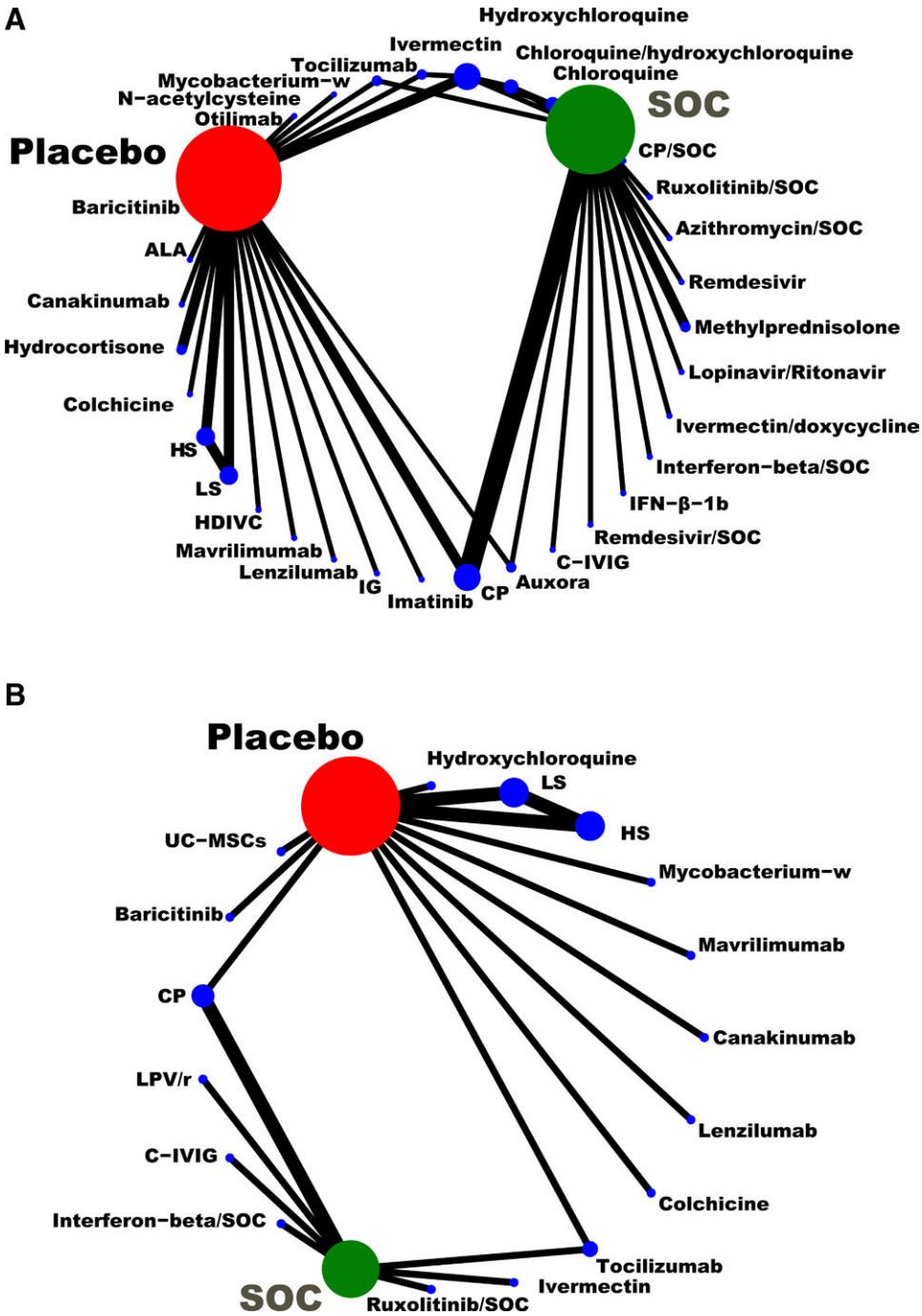


Figure 3. Network plot of eligible comparisons for all-cause mortality (A), and the ratio of treatment-emergent adverse events (B) for medications in patients with severe COVID-19. ALA= α-Lipoic acid, C-IVIG = hyperimmune anti-COVID-19 intravenous immunoglobulin, COVID-19 = coronavirus disease 2019, CP = convalescent plasma, IFN-β= interferon-beta, IG = immunoglobulin gamma, HDIVC = high-dose intravenous vitamin C, HS = high dosage sarilumab, LS = low dosage sarilumab, SOC = standard-of-care, UC-MSCs = human umbilical cord-derived mesenchymal stem cells.

HS, and mavrilimumab) or SOC for the ACM of severe COVID-19 infection (Fig. 4).

The Supplemental Figure S1, http://links.lww.com/MD/H538 presented the ranking for the ACM of medications in severe COVID-19 patients based on cumulative probability plots and SUCRA.

3.5. Safety outcomes

In the safety outcome, data from 19 studies[18,20,21,23,26–29,34,38–40,48,50–54,56]were merged for analysis (Supplemental Table S2, http://links.lww.com/MD/H537). We found that colchicine (OR 2.77, 95% CI 1.03-7.42) and IFN-β/SOC (OR 18.98, 95% CI 1.70-211.84) seemed to increase the risk of TEAEs when compared with the SOC group. Moreover, IFN-β/SOC (OR 19.00, 95% CI 2.36-153.10) were associated with the increase of TEAEs when compared with placebo. We found no significant difference in TEAEs in other 15 medications for severe COVID-19 patients when compared with SOC or placebo (Fig. 5).

The figure of SUCRA showed that baricitinib had the highest cumulative probability (SUCRA: 88.3%) becoming the best intervention in TEAEs, followed by LPV/r (SUCRA: 78.8%), SOC (SUCRA: 76.0%), lenzilumab (SUCRA: 72.6%),

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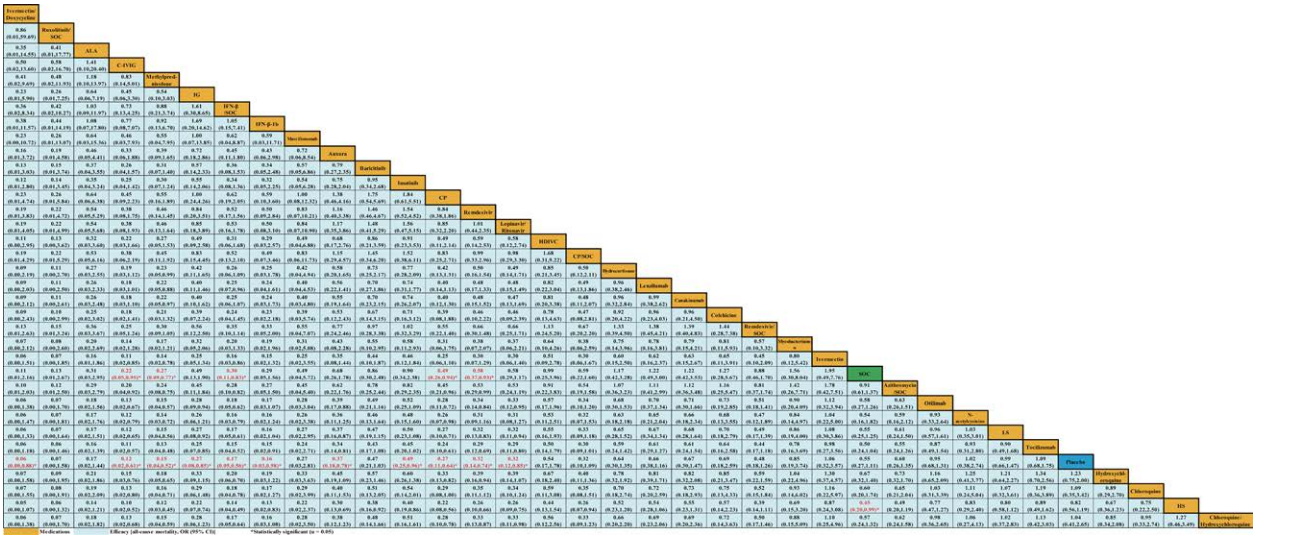


Figure 4. Network meta-analyses of the relative efficacy and safety of medications for all-cause mortality among patients with severe COVID-19 infection. The red font and asterisk indicated comparisons that were statistically significant. ALA= α-Lipoic acid, C-IVIG = hyperimmune anti-COVID-19 intravenous immunoglobulin, CI = confidence interval, COVID-19 = coronavirus disease 2019, CP = convalescent plasma, HDIVC = high-dose intravenous vitamin C, HS = high dosage sarilumab, IFN-β=interferon-beta, IG = immunoglobulin gamma, LS = low dosage sarilumab, OR = odds ratio, SOC = standard-of-care.

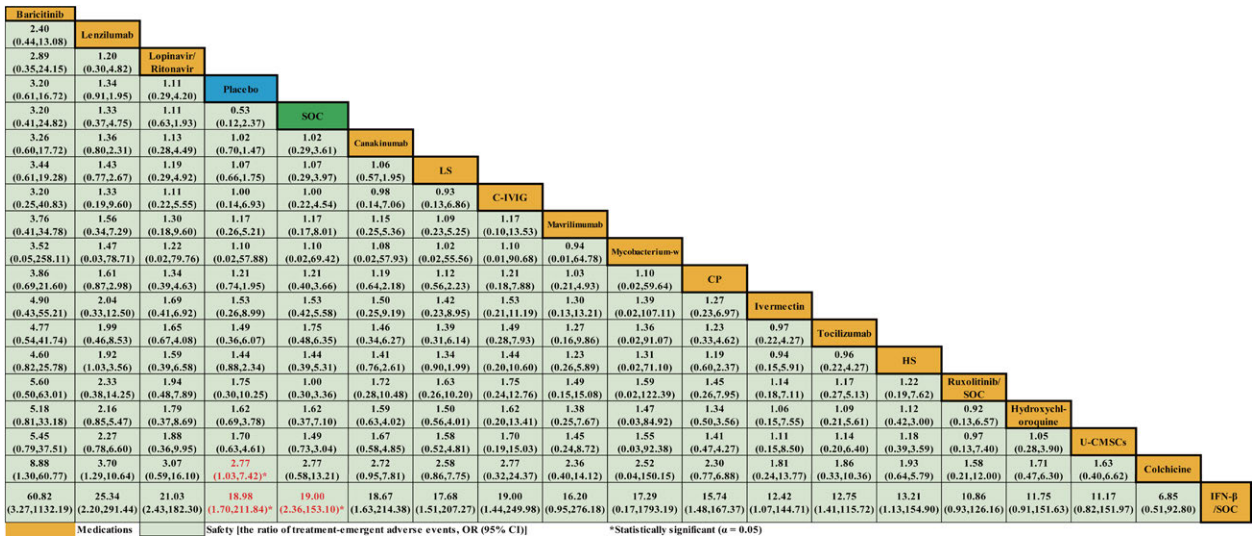


Figure 5. Network meta-analyses of the relative efficacy and safety of medications for the ratio of treatment-emergent adverse events among patients with severe COVID-19 infection. The red font and asterisk indicated comparisons that were statistically significant. C-IVIG = hyperimmune anti-COVID-19 intravenous immunoglobulin, CI = confidence interval, COVID-19 = coronavirus disease 2019, CP = convalescent plasma, HS = high dosage sarilumab, IFN-β=interferon-beta, LS = low dosage sarilumab, OR = odds ratio, SOC = standard-of-care, UC-MSCs = human umbilical cord-derived mesenchymal stem cells.

C-IVIG (SUCRA: 68.5%), tocilizumab (SUCRA: 63.6%), placebo (SUCRA: 53.1%), ivermectin (SUCRA: 52.3%), canakinumab (SUCRA: 51.9%), mycobacterium-w (SUCRA: 50.1%), LS (SUCRA: 49.3%), mavrilimumab (SUCRA: 47.1%), ruxolitinib/SOC (SUCRA: 46.8%), CP (SUCRA: 44.5%), HS (SUCRA: 31.0%), HCQ (SUCRA: 29.5%), human umbilical cord-derived mesenchymal stem cells (SUCRA: 29.1%), colchicine (SUCRA: 14.2%), and IFN-β/SOC (SUCRA: 3.2%) (Supplemental Figure S2, http://links.lww.com/MD/H539).

3.6. Evaluation of inconsistency

According to the inconsistency test (Table 2), no significant inconsistency or qualitative difference was available for the ACM and TEAEs. Thus, the consistency hypothesis was accepted in this NMA.

Table 2

The evaluation of inconsistency for the efficacy and safety of medications among severe COVID-19 patients.

Network meta-analysis	Number of dimensions	Chi² value	*P* value
All-cause mortality	34	2.37	.668
The ratio of treatment-emergent adverse events	18	0.69	.407

COVID-19 = coronavirus disease 2019.

FOI 26-3132 - Document 5

3.7. Sensitivity analysis and publication bias

We analyzed the potential sources of heterogeneity or inconsistency by using subgroup and meta-regression analyses. Univariable meta-regression and subgroup analyses indicated that there were heterogeneous sources (such as DS, blinding and RRB) for the ACM ($P < .05$) (Fig. 6A). Whilst the SS and RRB were the heterogeneity source of TEAEs based on the sensitivity analysis ($P < .05$) (Fig. 6B).

None of the funnel plots of outcomes (ACM and TEAEs) indicated a significant asymmetry (Supplemental Figure S3, http://links.lww.com/MD/H540).

4. Discussion

This study was based on 48 RCTs, which included 9147 severe patients randomly assigned to 35 medications or SOC or placebo. Our NMA indicated that 12 medications (i.e., ivermectin/doxycycline, C-IVIG, methylprednisolone, IFN-β/SOC, IFN-β-1b, CP, remdesivir, LPV/r, HS, IG, auxora, and imatinib) were efficacious for the therapy of severe COVID-19 infection based on the controls of SOC or placebo. For a decrease in ACM, on the one hand, C-IVIG, methylprednisolone, IFN-β/SOC, HS, remdesivir, and CP were more efficacious than SOC; on the other hand, the efficacy of ivermectin/doxycycline, C-IVIG, methylprednisolone, IFN-β/SOC, IFN-β-1b, CP, remdesivir, LPV/r, IG, auxora, and imatinib were superior to placebo in all 35 drug interventions. From this, we can infer that there is a difference in the findings of medications for severe COVID-19 patients from different control conditions in RCTs.

Curiously, our findings differed from our earlier pilot studies,[14,60] which have only indicated 2 effective medications (i.e., IG and methylprednisolone) in the decreased ACM for severe COVID-19 patients. In addition, the CP group showed lower TEAEs than the placebo for severe COVID-19 infection in our earlier NMAs.[14,60] These inconsistencies may be due to an increase in sample size in the updated NMA. The study extended the previous work that provided a reference for selecting the medication for patients with severe COVID-19 infection.[61] The present study raises the possibility that we cannot completely discount the efficacy of other medications for severe COVID-19 infection in clinic. So far, maybe it's because there was inadequate evidence of a benefit in ACM for them. In either case, it is noteworthy that we must fully consider its efficacy and safety when the medications are used in treating severe COVID-19 patients. Some medications (e.g., tocilizumab, otilimab, and mavrilimumab) might be a selection of combination treatment for COVID-19 infection.[62,63]

It is particularly worth mentioning that host factors, including age, prior-medical history, demographics, and medical facility, may be key determinants of disease severity and progression.[64] Similarly, the above factors are associated with the drug treatment effect of COVID-19 infection. Several studies have reported that aging and prior-medical history (such as cancer or chronic kidney/liver/lung diseases) might be 2 prominent impact factors for medical therapy from COVID-19.[65,66] Kopel *et* al suggested that some demographics factors (such as gender, ethnic group and socioeconomic status) were associated with the therapy of COVID-19 infection.[67,68] In another study in India, Sarkar et al[69] reported that different strains of SARS-CoV-2 were associated with the efficacy of antiviral drugs. It has also been suggested that medical facilities may be an impact factor for the COVID-19 treatment.[70] We have considered these impact factors in the overall therapy for COVID-19 patients during this NMA design. To reduce the impact of age, we included all of studies based on patient age greater than or equal to 16 years. As is well known, the design principles of RCTs are randomization, control, blinding, and repetition.[71] According to the 48 RCTs data analysis of this NMA, we confirmed that age, prior-medical history, gender and racial ratios for participants were balanced between experimental and control groups ($P > .05$). In view of this, we suggest that the effects of age, prior-medical history, gender and racial ratios in the findings are small in this NMA study. This might present one of the main strengthens of this NMA. Unfortunately, the description of most studies was insufficient in the gender-specific, different strains and medical facilities for COVID-19 therapy in previous RCTs. We could not explore the efficacy of drugs treatment from the gender-specific or different strains as well as the impact from medical facilities for COVID-19 therapy.

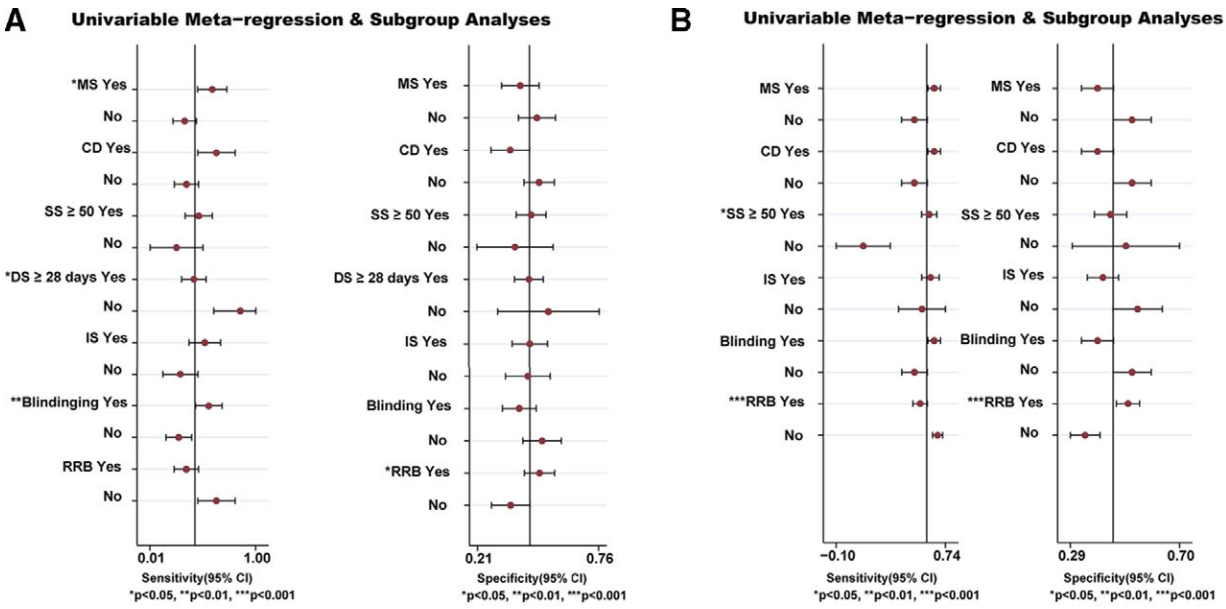


Figure 6. Meta-regression and sensitivity analyses for the efficacy and safety of medications in patients with severe COVID-19 infection. (A) all-cause mortality. (B) the ratio of treatment-emergent adverse events. CD = crossover design, COVID-19 = coronavirus disease 2019, DS = duration of study, IS = industry sponsorship, MS = multicenter study, RRB = risk of reported bias, SS = sample size.

4.1. Efficacy of current medications

This study supported evidence from previous observations.[72,73] As we know, intravenous immunoglobulin has already been validated as an effective antiviral drug for treating COVID-19, SARS and Middle East respiratory syndrome.[20,74,75] This is consistent with our results (i.e., C-IVIG, HS, and IG). Recent evidence suggested that anti-cytokine effects, inhibition of complement activation, and down-regulation of B and T cells' functions by C-IVIG can prevent organ failure and subsequent mortality in severe COVID-19 patients.[20] Our findings also confirmed that sarilumab could prevent patients with COVID-19 from progressing to death. Sarilumab is a fully human antibody against the interleukin (IL)-6 receptor, which can rapidly lower C-reactive protein and mediate COVID-19 clearance.[76] Prolonged glucocorticoid treatment is associated with improved outcomes of acute respiratory distress syndrome.[77] Several reports have also shown that treatment with methylprednisolone could significantly reduce the risk of death among patients with acute respiratory distress syndrome.[78] This also accords with our observations, which suggested that methylprednisolone was associated with decreased ACM in severe COVID-19 patients.

The SUCRA indicated that ivermectin/doxycycline was the highest ranked intervention with a SUCRA of 0.821. Previous studies showed that ivermectin or doxycycline is effective in treating COVID-19 patients.[79,80] We attribute this to a couple of reasons. First, ivermectin or doxycycline might possess antiviral as well as immunomodulatory activity.[79,80] Second, they might have anti-inflammatory and immunomodulatory agents and can curb over-reacting innate and cellular immune responses.[81] One possible implication may be that the ivermectin/doxycycline is an excellent selection for treating severe COVID-19 patients. Of note, based on the result of meta-regression analysis on the heterogeneity (such as DS, blinding, and RRB), the present result may need further verification. Thus, statistical indications of clinical superiority in this network analysis required careful interpretation.

Interestingly, we found that small-molecule protein kinase inhibitors (i.e., auxora and imatinib) were efficacious for the therapy of severe COVID-19 infection. Recent research has established that a calcium release-activated calcium channel inhibitor, such as auxora, can reduce the occurrence of COVID-19 death due to blocking the release of multiple pro-inflammatory cytokines, including IL-6. Additionally, evidence showed that imatinib might play its potentially antiviral and beneficial immunomodulatory role in severe COVID-19 patients.[82] These results further support those of previous studies.[25,83] Collectively, the present findings indicate that the small-molecule protein kinase inhibitors provide a new, and perhaps superior, avenue for the severe COVID-19 treatment. In addition, our results may provide a robust strategy for clinical combination therapy among severe COVID-19 patients.

We have demonstrated that IFN-β/SOC, IFN-β-1b, remdesivir, LPV/r and CP were associated with a reduction of ACM in severe COVID-19 infection. Several lines of evidence suggested that IFN deficiency was a hallmark of severe COVID-19.[84] IFN might treat severe COVID-19 infection through adapted anti-inflammatory therapies that target IL-6 or TNF-β.[84,85] Data from prior studies suggested that remdesivir was a broad-spectrum antiviral activity against RNA viruses, which could improve the survival of COVID-19 infection due to inhibiting viral replication.[86] Recent studies reported that LPV/r displayed inhibitory activities against SARS-CoV-2 main protease and inhibited SARS-CoV-2 replication in Vero E6 cells.[87] Similarly to other antibodies therapy, CP can prevent the death of severe COVID-19 patients because of alleviating the inflammation and overreaction of the immune system through antibodies.[88] These findings further support the idea of our NMA study.

Conversely, no significant difference was found in other 21 medications (e.g., HCQ, α-Lipoic acid, hydrocortisone, otilimab, and mavrilimumab) for the ACM of severe COVID-19 infection when compared with SOC or placebo. This finding was contrary to previous studies which have suggested that some antiviral drugs (hydrocortisone, mavrilimumab, etc) were associated with decreased ACM in patients with COVID-19.[29,32,89,90] It is difficult to explain this result, but it might be related to the difference of participants' selection in differential studies. For instance, the findings of this NMA were based on a larger sample size (i.e., more participants were included). Correspondingly, most of the previous studies presented a smaller sample size.[29,32] The present study differed from earlier studies,[90,91] which selected subjects with all infection levels (i.e., mild, moderate and severe infections). Furthermore, prior studies were inadequate for the analyses stratified by different infection levels (i.e., non-severe and severe infection) in medications of COVID-19.[29,32,89,90] Prior studies suggested that COVID-19 patients at different infection levels often led to different outcomes of treatment.[92] Another possible explanation for this was that we compared the efficacy and safety of SOC, which existed the bias due to the differential SOC of every country (i.e., the SOC is not standardized) except for the reasons given above.[21,23,45] The present study raised the possibility that our findings might be beneficial to guiding the selection of drug interventions for clinicians in severe COVID-19 patients.

4.2. Safety of current medications

In terms of safety, we summarized the TEAEs. We found that colchicine and IFN-β/SOC were only associated with the TEAEs of severe COVID-19 patients in this study. Recent meta-analysis studies and large-scale RCTs[45,46,92] seemed to be consistent with our findings, which identified most pharmacological treatments had a good safety in treating severe COVID-19. However, as mentioned in the present study, colchicine and IFN-β/SOC should be chosen cautiously in treating severe COVID-19 patients based on safety. Colchicine might increase the TEAEs in treating patients with severe COVID-19 infection. It is difficult to explain this result, but it might be related to the toxicity (e.g., gastrointestinal mucosal damage) in the case of colchicine treatment.[93] In addition, it is possible that the use of IFN-β in combination with SOC was associated with increased TEAEs in treating severe COVID-19, which should be weighed in all future IFN-β studies.[94]

In summary, clinicians might need to select treatment regimens based on the ranks of efficacy and safety (i.e., SUCRA) when medications are used in treating severe COVID-19 patients. Additionally, it should be reminded that further studies, which reduce the effects of SS and RRB, will need to be undertaken based on the result of sensitivity analysis.

Some limitations constrained this study. Firstly, included studies might be small in this NMA, which should be considered when interpreting the findings. Secondly, the published data we extracted included only 2 types of outcomes, some important outcomes such as discharge ratio, clinical improvement ratio, and the ratio of virological cure, were not analyzed. Thirdly, although we did our best to include all available RCTs, we cannot eliminate the possibility of missing data. Fourthly, some nodes in our network included only a few trials. The sample size of actual head-to-head trials was very small. Hence, comparative efficacy and safety between interventions was frequently based on indirect comparisons. Finally, the sensitivity analysis showed that there were several heterogeneity sources, which may conceal or exaggerate the effect size of this network analysis. Further large-scale RCTs, which control these confounding factors, will need to be undertaken to verify our findings. Though there are still many shortcomings in our research, it is certain that the prevention

FOI 26-3132 - Document 5

and therapy of COVID-19 is set to change for the better in the future.

5. Conclusions

In conclusion, this NMA demonstrated that ivermectin/doxycycline, C-IVIG, methylprednisolone, IFN-β/SOC, IFN-β-1b, CP, remdesivir, LPV/r, IG, HS, auxora, and imatinib were effective for treating severe COVID-19 patients. There may be a difference in the findings of medications for severe COVID-19 patients from different control conditions (i.e., placebo and SOC) in RCTs. We found that most medications were safe in treating severe COVID-19. The present NMA reported uncertain estimates on the efficacy and safety of medications in the severe COVID-19 treatment. Maybe it's because there was inadequate evidence of a reduction in ACM and the absence of TEAEs. However, this study had 2 strengths. One was that a comprehensive meta-analysis strategy was used to reduce the risk of publication bias. The other was that the SUCRA was used to assess possibly the best intervention.

Despite these limitations, to date, the present findings might represent the most comprehensive meta-analysis of the available evidence for severe COVID-19 infection. Future guidelines and decision-making treatment plan should consider these results for the severe COVID-19 treatment. Importantly, the government, academia and researchers should collaborate to develop more large-scale RCTs studies and further estimate the efficacy and safety of treatment interventions on mortality, virological and clinical outcomes for different levels of infection with COVID-19.

Author contributions

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Formal analysis: Cheng Qing Lin, Zhao Gang.
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Supervision: Jia Qing Jun, Fang Zi Jian.
Writing – original draft: Cheng Qing Lin.
Writing – review & editing: Cheng Qing Lin, Zhao Gang.

Acknowledgments

We acknowledge with gratitude all study authors who responded to our data requests. We thank many researchers who sent information for our previous reviews on which this report was built. This work is supported by the Basic Public Welfare Research Project of Zhejiang Province (grant number: LGF21H260007), the Medical Science and Technology Project of Zhejiang Province (grant numbers: 2020PY064 and 2021PY065), and the Key Medical Discipline construction project (Disinfection and Vector Control) of Hangzhou.

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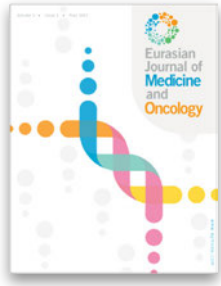
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FOI 26-3132 - Document 5

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EJMO

DOI: 10.14744/ejmo.2021.16263
EJMO 2021;5(1):63–70



Research Article

A Comparative Study on Ivermectin-Doxycycline and Hydroxychloroquine-Azithromycin Therapy on COVID-19 Patients

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Abstract

Objectives: We investigated the outcomes of Ivermectin-Doxycycline vs. Hydroxychloroquine-Azithromycin combination therapy in mild to moderate COVID19 patients.

Methods: Patients were divided randomly into two groups: Ivermectin 200µgm/kg single dose + Doxycycline 100mg BID for ten days in group A, and Hydroxychloroquine 400mg for the first day, then 200mg BID for nine days + Azithromycin 500mg daily for five days in group B (Control group). RT-PCR for SARS-CoV-2 infection was repeated in all symptomatic patients on the second day onward without symptoms. Repeat PCR was done every two days onward if the result found positive. Time to the negative PCR and symptomatic recovery was measured for each group.

Results: All subjects in Group A reached a negative PCR, at a mean of 8.93 days, and reached symptomatic recovery, at a mean of 5.93 days, with 55.10% symptom-free by the fifth day. In group B, 96.36% reached a negative PCR at a mean of 9.33 days and were symptoms-free at 6.99 days. In group A 31.67% of patients expressed symptoms caused by medication, this was 46.43% in group B.

Conclusion: The combination therapy of Ivermectin-Doxycycline showed a trend towards superiority to the combination of Hydroxychloroquine-Azithromycin for mild to moderate COVID19 disease.

Keywords: Azithromycin, Bangladesh, COVID-19, Doxycycline, Hydroxychloroquine, Ivermectin, randomized controlled trial (RCT)

Cite This Article: *Mohiuddin Chowdhury ATM, Shahbaz M, Karim MR, Islam J, Dan G, He S. A Comparative Study on Ivermectin-Doxycycline and Hydroxychloroquine-Azithromycin Therapy on COVID-19 Patients. EJMO 2021;5(1):63–70.*

Coronavirus disease 2019 (COVID-19) is a global pandemic declared by the world health organization (WHO). Over ninety million people have already been infected by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), and billions have been affected by the socioeconomic squeal. As SARS-CoV-2 is a novel virus, there are no proven treatment options yet. Early treatment before the disease becomes severe would be optimal. The

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Submitted Date: January 21, 2021 **Accepted Date:** February 15, 2021 **Available Online Date:** February 25, 2021
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treatment's current considerations include lopinavir/ritonavir, favipiravir, and remdesivir. These are currently in use in moderate to severe COVID-19 patients. Treatments for patients in the early stage of the disease with mild to moderate symptoms have not been well established. Studies have shown that Chloroquine and Hydroxychloroquine (HCQ) are not beneficial in moderate to severe COVID19 disease. However, they have become the current standard for mild to moderate and early disease states. Chloroquine has been shown as a potential suppressor of SARS-CoV-2 in an in vitro study.[1] Though many trials have established a good outcome in mild to moderate cases, unfortunately, chloroquine toxicity is a paramount concern.[2] HCQ, a less toxic derivative, has also been found to be effective.[3] Recently, an anti-parasitic drug Ivermectin has been described as highly effective in an in vitro study against SARS-CoV-2.[4] HCQ-Azithromycin combination therapy has also been shown to be a possibly effective combination therapy in the treatment of SARS-CoV-2.[5, 6] These two studies reported 100% and 83% recovery in the sixth and seventh day with a reduced hospital stay. Alam MT et al.[31] recently described an encouraging result in a case series of COVID19 patients with a combination of Ivermectin and Doxycycline. Till now, there has not been a randomized study of Ivermectin in patients with mild to moderate COVID19. Ivermectin is well-tolerated, less toxic, and has fewer adverse effects than HCQ. HCQ-Azithromycin combination therapy has also been mentioned in the "National guideline for COVID management 4.0" of Bangladesh as an initial therapy against COVID19. Due to drug complications and discouraging statements about HCQ treatment made by WHO, it is crucial to find an effective, economical alternative to HCQ. Therefore, we decided to investigate the efficacy of Ivermectin-Doxycycline combination therapy and compare it to the standard HCQ-Azithromycin treatment according to the "National guideline for COVID management 4.0" among the mild to moderate cases of COVID19 patients.

Methods

Ethical Consideration and Approval, Informed Consent

This study was approved by the Ethical committee of Chakoria Upazilla Health Complex, Chittagong, Bangladesh. UHC/Chakoria/Ethical Approval/2020/01 (15-04-20). The purpose of the research was explained to participants. Once verbal consent was understood and agreed upon, a written form was given. This was done to get informed consent and legal identification. The individuals who gave consent were enrolled in this study.

Study Population and Data Collection

Patients (16 years to 80 years of age) tested positive for SARS-CoV-2 infection by Real-time polymerase chain reaction (RT-PCR) at Chakoria Upazilla Health Complex, Cox's Bazar, Bangladesh, from second may to the fifth June 2020, were initially included in this study, including those with and without the symptoms. The PCR analysis of the collected samples was performed in Cox's Bazar Medical College. All patients received a full evaluation, including a history of current illness, comorbid condition, and associated complaints. Patients with unstable comorbid conditions like bronchial asthma, chronic obstructive pulmonary disease (COPD), ischemic heart disease (IHD), uncontrolled diabetes mellitus (DM), advanced renal and hepatic disease, carcinoma, hospitalized, and immuno-compromised patients were excluded. Patients were examined for oxygen saturation and only those with oxygen saturation of 95% or above who fit the outpatient treatment protocol for COVID19 were included. Patients with respiratory symptoms received chest radiographs. Those with normal or near-normal chest radiographs (up to 10% involvement) were included.

Randomization and Treatment Intervention

Randomization was done using an odd-even methodology and applied to registration numbers, consecutively in a 1:1 ratio, by the hospital registration office. Treatment was given, and the final enrolment was done by the attending physician (investigator). All the patients enrolled in this study were treated as outpatient department (OPD) patients.

For the study, patients were divided into two groups, as follows:

- Group A (n=60): Ivermectin 200µgm/kg single dose + Doxycycline 100mg BID for ten days.
- Group B (n=56): Hydroxychloroquine 400mg for the first day, then 200mg BID for nine days + Azithromycin 500mg daily for five days (considered as the control group).

Ivermectin dosing was determined by the case series performed by Alam MT et al., and HCQ dosing was decided as per "National guideline for COVID management 4.0".

All the subjects were provided with symptomatic treatment such as fever, headache, cough, and myalgia. Drug interactions and contraindications for each individual were considered carefully. The schedule of medication intake was adequately explained to each patient. Group A's instructions included that Ivermectin tablet (200µg/kg) single dose to be taken on an empty stomach an hour before a meal on the first day. Doxycycline 100 mg cap-

sule be taken twice daily after meal for ten days starting from day one. Group B's instructions included Hydroxychloroquine 400mg (two tablets of 200mg each) to be taken on the first day, then 200mg (one tablet) twice daily after meal for nine days. Azithromycin 500mg (one tablet of 500mg) to be taken once daily after meal for five days starting from day one. Patients were advised to self-isolate, take proper nutrition, hydration, and maintain a sanitary environment.

Repeat Nasopharyngeal and Throat Swab PCR

All subjects underwent repeat nasopharyngeal and throat swab PCR for SARS-CoV2 every other day until their PCR was negative. These repeat PCR tests began on the fifth day after taking the medication for subjects who began the study and remained symptom-free. The PCR repeat testing began on the second symptom-free day onward for subjects who began the study with symptoms or developed symptoms. The investigators had telephone contact with all the subjects every three days throughout the study to determine any therapy's adverse effects. A re-evaluation PCR was performed after seven days following the first negative PCR.

Endpoints were a negative PCR and resolution of symptoms. The duration from the first day of drug intake to the negative PCR was counted as the recovery period. The duration from the first day of drug intake to the disappearance of symptoms was counted as the period to symptomatic recovery. "Adverse effects" were determined by the existence of the pharmacological side effects of the particular drug during treatment. A detailed history of adverse effects (other than previous disease symptoms) experienced by each participant was collected during the follow-up sample collection. An asymptomatic participant presented with no symptom of COVID-19 and remained the same until the negative PCR.

Data Analysis and Statistics

Data were presented as mean±standard deviation, and statistical analysis was done by Graph pad Prism software. Column analysis was done to find the mean with the standard deviation in each group. T-test was done to see the significance between the values where needed. $P<0.05$ was considered statistically significant.

Results

This study was completed in a pre-determined period from May second to June fifth, 2020, and 181 patients tested positive for SARS-CoV-2 infection in that period. 42 patients had comorbid conditions (some required hospitalization) that might have affected the recovery time, and 14 patients were unwilling to participate in the study. Nonetheless, 9 patients did not show-up (3 from group A and 6 from group B) for the follow-up sample collection, so these were excluded, and 116 patients were finally included in the analysis (Fig. 3).

Demographic Characteristics of the Study Subjects

As shown in Table 1, the total number of patients was 116; male 84 and female 26, age 16 to 80 years, and mean age (33.94±14.12 years). Group A (Ivermectin + Doxycycline): male 43 (71.67%), female 17 (28.33%), age 35.72±15.1 years; males 37 years and female 32.88 years. Group B (Hydroxychloroquine + Azithromycin): male 47 (83.93%), female 9 (16.07%), age 31.91 years; male 31.35, and female 34.5 years. (Fig. 1 a, b) Among the total, 91 (78.45%) were symptomatic, and 25 (21.55%) were asymptomatic patients with contact history. These were 49 (81.67%) and 11 (18.33%) in group A, 42 (75%) and 14 (25%) in group B.

Recovery Rate and Mean Recovery Duration Between Groups

In group A, recovery to negative PCR rate was 100% (60/60). The mean recovery duration to negative PCR was 8.93 days (8 to 13 days). 41 (63.3%) of patients had no new complaints other than their presenting symptoms. New symptoms that may have been attributed to adverse drug effects included

Table 1. Baseline characteristics of the study group patients.

Parameters	
Number of patients (n)	116
Male	90 (77.58)
Female	26 (22.41)
Group A (n, %)	60
Group A Male (n, %)	43 (71.67)
Group A Female (n, %)	17 (28.33)
Group B (n)	56
Group B Male (n, %)	47 (83.93)
Group A Female (n, %)	9 (16.07)
Age (in years)	33.94±14.12 (8 to 80 Years)
Symptomatic (n, %)	91 (78.45)
Asymptomatic (n, %)	25 (21.55)
Agegroup A (in years)	35.72±15.1
Male	37.14±14.72
Female	32.88±16.2
Symptomatic (n, %)	49 (81.67)
Asymptomatic (n, %)	11 (18.33)
Agegroup B (in years)	31.91±12.72
Male	31.35±12.95
Female	34.5±11.74
Symptomatic (n, %)	42 (75)
Asymptomatic (n, %)	14 (25)

FOI 26-3132 - Document 5

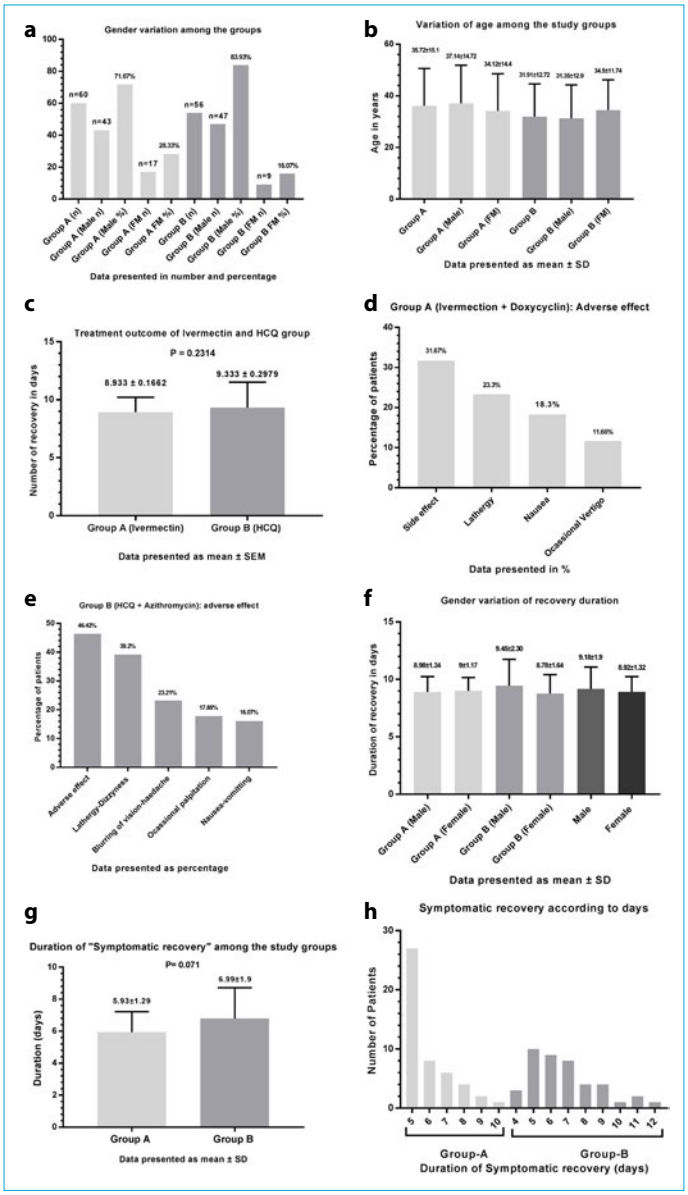


Figure 1. (a) Number of patients according to gender among the groups. **(b)** Gender variation of age among the study groups (data presented as mean±SD). **(c)** Recover duration of Ivermectin-Doxycycline and HCQ-Azithromycin group; note: the difference between the groups' recovery duration is not statistically significant P=0.231. **(d)** Adverse effect expressed by the patients of group A. **(e)** Adverse effect experienced by the patients of group B. **(f)** Variation of the recovery duration according to the gender; note: males in group B and males as gender, in general, have a longer recovery period than the females. **(g)** Duration of the symptomatic recovery (in days) among the groups. The difference between the duration among groups is not significant P=0.071. **(h)** Subgroup analysis of the recovery duration among the groups. Note: group A has a higher number of symptomatic recoveries in the early days; this indicates a better efficacy of Ivermectin-Doxycycline therapy.

lethargy in 14 (23.3%), nausea in 11 (18.3%), and occasional vertigo in 7 (11.66%) of patients (Fig. 1c).

In group B, out of 56 patients, two male patients were referred to a tertiary hospital. They did not recover to a negative PCR as part of the study. Therefore, the recovery rate to negative PCR was 96.36% (54/56). The mean duration of recovery to negative PCR was 9.33 days (5 to 15 days). 30 (53.57%) of the patients had no new complaints other than their presenting symptoms. Fresh symptoms that were recognized as an adverse effect of HCQ included 13(23.21%) with mild blurred vision and headache; 22 (39.2%) with increased lethargy and dizziness, 10 (17.85%) with occasional, mild palpitation, and 9 (16.07%) with nausea and vomiting (Fig. 1e).

Difference in Recovery to Negative PCR between Groups

The difference between group A and group B recovery to negative PCR duration was not statistically significant in unpaired t-test, p=0.2314 (Fig. 1c). Subgroup analysis of the recovery duration: male 9.18±1.90 days and female 8.92±1.32 days, p=0.515; in group A male 8.907±1.342 days and female 9±1.173 days, p=0.44; and in group B male 9.18±1.90 days and female 8.92±1.32 days, p=0.407. The recovery duration of both group males and females were not significant, p=0.18 and 0.69, respectively (Fig. 1f).

The mean duration of symptomatic recovery was 5.93 days (5 to 10 days) in group A and 6.99 days (4 to 12 days) in group B (Fig. 1g). This difference in time to symptomatic recovery between group A and group B is not statistically significant, p=0.071 (Fig. 1g). In group A, over half of the subjects had become symptom-free by five days 27 (55.10%), with the remaining subjects becoming symptom-free on day six (16.32%), day seven (12.24%), day eight (8.16%), day nine (4%), and day ten (2.04%) (Fig. 1h). In group B, recovery was slower with subjects becoming symptoms free on fourth day 3 (7.14%), the fifth day 10 (23.8%), sixth day 9 (21.43%), seventh day 8(19.04%), eighth & ninth day 4 (9.52% each), eleventh day 2 (4.76%), and tenth and twelfth day (2.38% each) (Fig. 1h).

Mean Duration of Time to Negative PCR between Groups

In the secondary analysis of subjects who began the study with symptoms, the mean duration of time to negative PCR was 9.061 days in group A and 9.738 days in group B. This was not statistically significant in the unpaired t-test, p=0.0714. The mean duration of time to becoming negative PCR of patients without symptoms was 8.364 days in group A and 7.917 days in group B, which was not statistically significant in unpaired t-test, p=0.443 (Fig. 2b). Further analysis showed the highest recovery was achieved on the eighth day among group A patients in case of both asymptomatic (n=11) and symptomatic (n=49) patients,

8 (72.72%) and 22 (44.89%), respectively (Fig. 2 c, d). This recovery was relatively slower in group B. On the sixth day 3 (7.5%), seventh day 1 (2.5%), eighth day 9 (22.5%), ninth and tenth day 8 (20%) each, eleventh and twelfth day 4 (10%) each, thirteenth day 1 (2.5%), and fourteenth day 2 (5%) in group B patients with symptoms (n=40). Among asymptomatic patients (n=14), this was 1 (7.5%) on the fifth day, 2 (14.2%) individually on the sixth, seventh, eighth, tenth day, 4 (28.57%) on the ninth day, and 1 (7.1%) on the eleventh day (Fig. 2 c, d). No significant difference in the recovery duration was found in the subgroup analysis of the recovery duration according to the study groups' age. 61 to 70 years in group A had the longest recovery duration, 9.5±2.12, and 71 to 80 years was the shortest 8days. In group B, this was 11.71±2.48 days in the 41 to 50 years and 8.37±2.44 days in the 10 to 20 years age group (Fig. 2 e).

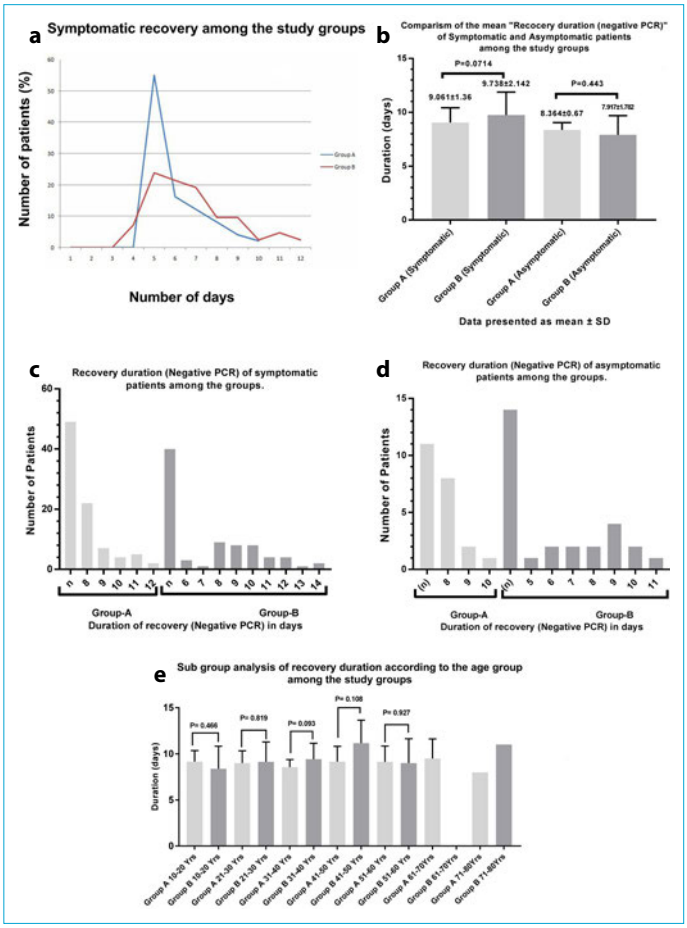


Figure 2. (a) Comparison between the symptomatic recoveries among the study groups. Note: group A (Ivermectin-Doxycycline) showed a pick recovery on the 5th day. In group B (HCQ-Azithromycin), the symptomatic recovery started relatively earlier on the 4th day but had a slow trend. **(b)** The mean duration of recovery (beginning of the treatment to the negative PCR) of the patients with and without symptoms among groups A& B was not statistically significant in the subgroup analysis. **(c)** Subgroup analysis of recovery duration of the symptomatic patients among the study groups. Maximum numbers of negative PCR were achieved on the 8th day in both groups. Group A 22 (44.89%) and group B 9 (22.5%). The recovery rates are faster in group A, though relatively earlier but slow in group B. **(d)** Subgroup analysis of the asymptomatic patients' recovery duration. Most group A patients gained viral clearance on 8th day 8 (72.72%). Not: first PCR was done on the 5th day. In group B, 1(7.1%) was recovered on the 5th day and 2 (14.2%) on the 6th day. The highest recovery was observed on the 9th day 4 (28.57%). **(e)** Subgroup comparison of the recovery duration according to age.

Discussion

The COVID-19 pandemic in Bangladesh is part of the coronavirus worldwide pandemic disease caused by a newly discovered coronavirus. It was initially called novel coronavirus and later named SARS-CoV-2 due to its similar characteristics with SARS-CoV-1.[7-9] The treatment methods for COVID-19 are emerging and rapidly evolving because of ongoing research being done worldwide by a record number of investigators. Due to each medical and research facility's uniqueness, the approach to patient care with COVID-19 varies from institution to institution in Bangladesh. Many patients with mild to moderate disease were treated with HCQ and Azithromycin. New concerns about HCQ has led us to seek alternatives with shorter recovery time and better tolerability. Thus, we have undertaken a comparative therapeutic analysis, comparing these standard drugs with Ivermectin and Doxycycline.

In this randomized treatment study of groups A and B, the presenting symptoms of the COVID19 patients were fever, cough, sore throat, weakness, chest discomfort, breath-

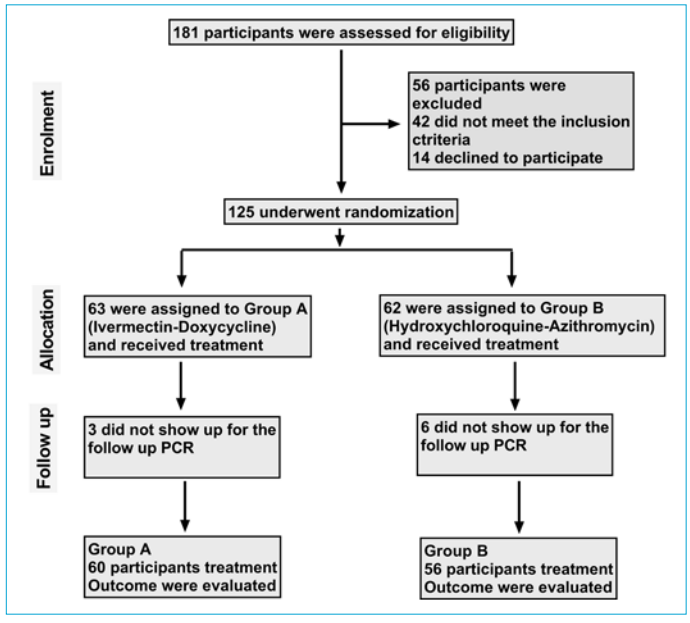


Figure 3. Flow diagram of randomization and treatment assignment of the participants.

ing difficulty, diarrhea, myalgia, and abdominal pain. To avoid the recovery duration's influence, we solely selected the cases devoid of severe comorbidities. The difference in recovery to negative PCR duration was not significant (p=0.231) among the two groups. The mean recovery duration is shorter, 8.933 days in group A than in group B, 9.33 days (Fig. 1 c). Also, group A had a better outcome ratio of 100% (60/60) recovery to negative PCR compared to that of group B 96.36% (54/56).

HCQ has decades of treatment use as an immunomodulator.[10] At present, it has been the topic of discussion concerning its potential use to treat patients with COVID-19.[11] It is thought that the effect of HCQ results in the selective killing of the infected cells. Therefore, it may accelerate viral clearance in COVID-19.[13] Some studies showed that severe deterioration in some patients with COVID-19 had been closely associated with dysregulated and excessive cytokine release termed "cytokine storm."[14, 15] HCQ was found to inhibit SARS-CoV-2 infection in vitro and significantly decrease cytokine production, especially the pro-inflammatory cytokines.[16] Correspondingly, Azithromycin is a macrolide group of antibiotics. It has been studied as part of possible treatment of COVID-19 combined with HCQ and has been reported to add benefit.[17, 18] However, a recent report failed to establish whether it has any antiviral activity or any synergistic activity with HCQ in the treatment of COVID-19.[19] Therefore, a further comparative study can enhance the significance of HCQ- Azithromycincombination therapy.

On the other hand, Ivermectin is a relatively safe and well-tolerated anti-parasitic drug that can inhibit nuclear transport activity.[20] Recently, in-vitro studies have shown its function against SARS-CoV-2.[21, 22] A recent report suggested that Ivermectin reduces mortality rates in hospitalized patients with COVID-19.[23] However, it is unknown if antiviral levels are attainable while using known dosing regimens of Ivermectin therapy in patients with COVID-19.[24, 25] Thus, it is vital to investigate Ivermectin's dose regimens for COVID-19 treatment or to determine if there is appropriate synergism using combination therapy with another drug. Also, Doxycycline is a tetracycline class of antibiotics with a long history of clinical use.[26] The efficacy and tolerability of Ivermectin and Doxycycline were established in combination with an earlier study to treat onchocerciasis.[27] Several recent studies have suggested a therapeutic role of Doxycycline against COVID-19.[28, 29]

In our study, the difference in recovery to become symptom-free was not statistically significant (Fig. 1c). Nevertheless, the Ivermectin group showed better symptomatic recovery than the HCQ group (Fig. 2 a-d). According to the age among study groups, the difference was not statistically significant (Fig. 2 e). Ivermectin-Doxycycline combination expressed an earlier and faster relief of COVID features (Fig. 1 g) and viral clearance than the HCQ-Azithromycin combination. However, the mean recovery duration is not statistically significant (Fig. 1c). In the Ivermectin-Doxycycline group a greater number of patients gained faster symptomatic recovery than that of the HCQ group (Fig. 1h] This suggests Ivermectin-Doxycycline may have better efficacy in reducing the COVID-19 symptoms than HCQ-Azithromycin therapy.

The Ivermectin-Doxycycline group had better patient compliance and fewer adverse effects compared to the HCQ-Azithromycin group (Fig. 1 d, e). The adverse effects of HCQ in our study are similar to others.[30, 31] The sex difference was also examined, but there were no significant differences between males and females in this study (Fig. 1 f). According to this study, the Ivermectin-Doxycycline treatment regimen was well tolerated, and effective treatment for mild to moderate degrees of SARS-CoV-2 infection. Not only concerning the time to become symptom-free and the viral clearance, but also in terms of safety, side-effect profile, and compliance the Ivermectin-Doxycycline combination is superior to HCQ-Azithromycin therapy for mild to moderate degrees of COVID-19 patients. We strongly believe that increasing the dose and the duration of Ivermectin treatment will further benefit in reducing the recovery period of COVID19 infection beyond that which was seen in our study. This will also prevent disease progression and morbidity in COVID-19 patients.

Our study has limitations, and these include relatively small sample size, the dose of Ivermectin, and case selection. The outcome may be biased by factors like disease severity, lack of cooperation of some participants, and unknown comorbidity.

Conclusion

Researchers have suggested different drug combination therapies for COVID19. According to our study, the Ivermectin-Doxycycline combination therapy has better symptomatic relief, shortened recovery duration, fewer adverse effects, and superior patient compliance compared to the Hydroxychloroquine-Azithromycin combination. Based on this study's outcomes, the Ivermectin-Doxycycline combination is a superior choice for treating patients with mild to moderate COVID-19 disease. Despite this study's limitation, we tried to select our study group patients without any major or unstable comorbid condition as far as possible to avoid differences in treatment outcomes among the groups. Further study is required on a larger scale with an increase in Ivermectin treatment duration.

Disclosures

Acknowledgment: Alexis Lieberman, MD; Associate chief for Ambulatory Pediatrics and Director of the Adolescent Program at Albert Einstein Medical Center in Philadelphia, Pennsylvania, for his kind assistance in editing this manuscript.

The authors are tankful to all the doctors and staffs of the Chakoria Upazilla Health Complex, Department of Gastroenterology-First Affiliated Hospital of Xi'an Jiaotong University, and to Ministry of Health and Family welfare Bangladesh.

Other affiliation of Abu Taiub Mohammed Mohiuddin Chowdhury: Ministry of Health and Family welfare Bangladesh.

Ethics Committee Approval: The study was approved by the local ethical committee of Chakoria Upazilla Health Complex, Cox's Bazar, Bangladesh. Approval no: UHC/Chakoria/Ethical Approval/2020/01(15-04-20)ClinicalTrials.gov ID: NCT04434144.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – A.T.M.M.C.; Design – A.T.M.M.C.; Supervision – S.H.; Materials – M.R.K.; Data collection &/or processing – A.T.M.M.C., J.I., G.D.; Analysis and/or interpreta-tion – A.T.M.M.C. Literature search – M.R.K.; Writing – A.T.M.M.C.; Critical review – S.H.

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FOI 26-3132 - Document 5

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In Vitro Antiviral Activity of Doxycycline against SARS-CoV-2

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Received: 18 September 2020; Accepted: 30 October 2020; Published: 31 October 2020



Abstract: In December 2019, a new severe acute respiratory syndrome coronavirus (SARS-CoV-2), causing coronavirus disease 2019 (COVID-19), emerged in Wuhan, China. Despite containment measures, SARS-CoV-2 spread in Asia, Southern Europe, then in America and currently in Africa. Identifying effective antiviral drugs is urgently needed. An efficient approach to drug discovery is to evaluate whether existing approved drugs can be efficient against SARS-CoV-2. Doxycycline, which is a second-generation tetracycline with broad-spectrum antimicrobial, antimalarial and anti-inflammatory activities, showed in vitro activity on Vero E6 cells infected with a clinically isolated SARS-CoV-2 strain (IHUMI-3) with median effective concentration (EC_{50}) of 4.5 ± 2.9 µM, compatible with oral uptake and intravenous administrations. Doxycycline interacted both on SARS-CoV-2 entry and in replication after virus entry. Besides its in vitro antiviral activity against SARS-CoV-2, doxycycline has anti-inflammatory effects by decreasing the expression of various pro-inflammatory cytokines and could prevent co-infections and superinfections due to broad-spectrum antimicrobial activity. Therefore, doxycycline could be a potential partner of COVID-19 therapies. However, these results must be taken with caution regarding the potential use in SARS-CoV-2-infected patients: it is difficult to translate in vitro study results to actual clinical treatment in patients. In vivo evaluation in animal experimental models is required to confirm the antiviral effects of doxycycline on SARS-CoV-2 and more trials of high-risk patients with moderate to severe COVID-19 infections must be initiated.

Keywords: COVID-19; SARS-CoV-2; doxycycline; treatment; prophylaxis; antiviral; anti-inflammatory

1. Introduction

In December 2019, a new severe acute respiratory syndrome coronavirus (SARS-CoV-2), causing coronavirus disease 2019 (COVID-19), emerged in Wuhan, China [1]. Despite containment measures, SARS-CoV-2 spread in Asia, Southern Europe, then in America and in Africa. As of 9 October 2020, according to the treatment guideline of the National Institutes of Health (NIH), there is no Food and

FOI 26-3132 - Document 5

Drug Administration-approved antiviral treatment against SARS-CoV-2 [2]. Several drugs are tested in the context of the DisCoVeRy trial [3] and a controversial early treatment that associates a combination of hydroxychloroquine and azithromycin has been used worldwide with a reduced risk of hospitalization, a reduced risk of death and a shorter duration of viral shedding [4–10]. Conversely, some studies showed that treatment of mild-to-moderate or mild-to-severe COVID-19 with hydroxychloroquine alone or in combination with azithromycin did not improve clinical status or duration of viral shedding in comparison with standard care [11–15]. Identifying effective, low cost antiviral drugs with limited side effects which are affordable immediately, especially for emerging countries, is urgently needed. An efficient approach to drug discovery is drug repurposing that consists of evaluating whether existing approved drugs can be efficient against SARS-CoV-2.

Doxycycline is a second-generation tetracycline with broad-spectrum antimicrobial [16] and anti-inflammatory activities [17]. Additionally, doxycycline was approved as prophylaxis against malaria by the Food and Drug Administration in 1994 and has been used since 2006 at the dose of 100 mg/day by the French military forces deployed in malaria-endemic areas [18].

Doxycycline also shows antiviral activity in vitro. This tetracycline derivative significantly inhibited the replication of the vesicular stomatis virus in vitro [19] and that of the dengue virus by inhibition of NS2B-NS3 serine protease [20–22]. Doxycycline showed inhibition of entry and replication of Chikungunya virus in Vero cell at 11 μM [23]. Using the in-silico method, doxycycline might be a potential inhibitor of Crimean-Congo hemorrhagic fever virus nucleoprotein, an essential protein in virus replication [24]. Additionally, doxycycline inhibited the early-stage replication of the porcine reproductive and respiratory syndrome virus, which causes respiratory disease, with EC_{50} (median effective concentration) of 0.25 μg/mL (about 0.5 μM) [25]. The current study evaluated the antiviral effect of doxycycline against SARS-CoV-2.

Doxycycline could be an attractive option for the treatment of COVID-19 [26]. Therefore, the activity of doxycycline was assessed in vitro against a clinically isolated SARS-CoV-2 strain and was compared with the activity of chloroquine.

2. Results

The cytotoxicity evaluation of doxycycline and chloroquine showed that the CC_{50} values were >100 μM for 48 h. The CC_{50} value of chloroquine is consistent with those previously described [27,28]. The antiviral effects of doxycycline against the clinically isolated SARS-CoV-2 strain (IHUMI-3) were concentration-dependent.

The median effective concentration (EC_{50}) and 90% effective concentration (EC_{90}) for doxycycline were 4.5 ± 2.9 μM and 23.5 ± 16.5 μM, respectively (Figure 1). The EC_{50} and EC_{90} for chloroquine were 3.2 ± 1.8 μM and 13.9 ± 6.4 μM, respectively (Figure 2). The EC_{50} value for chloroquine is consistent with previous results on Vero E6 cells at MOI of 0.2 to 0.25 [27,29].

The ratios C_{max}/EC_{50} and C_{max}/EC_{90} in blood for doxycycline were estimated at 0.75 and 0.07, respectively after an oral administration of doxycycline 100 mg, at 2.21 and 0.41 after an oral administration of doxycycline 200 mg and at 1.14 and 0.21 after intravenous administration of 100 mg of doxycycline.

Our results demonstrated that doxycycline interacted at both entry and post-entry stages of the SARS-CoV-2 infection in Vero E6 cells (Figure 2).

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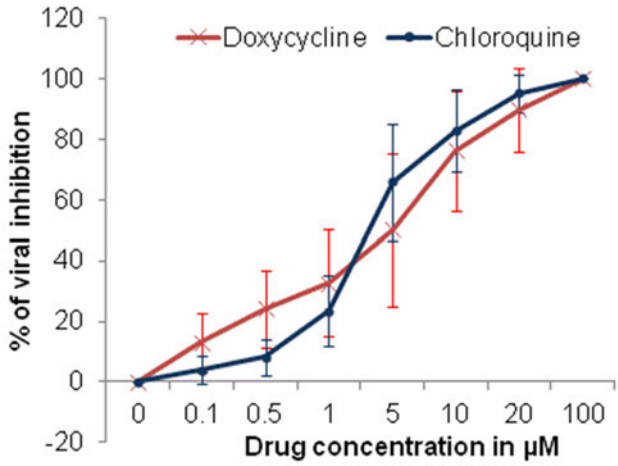


Figure 1. Comparative antiviral efficacy of doxycycline and chloroquine against SARS-CoV-2 infection in vitro (error bar represents standard deviation, 10 experiments).

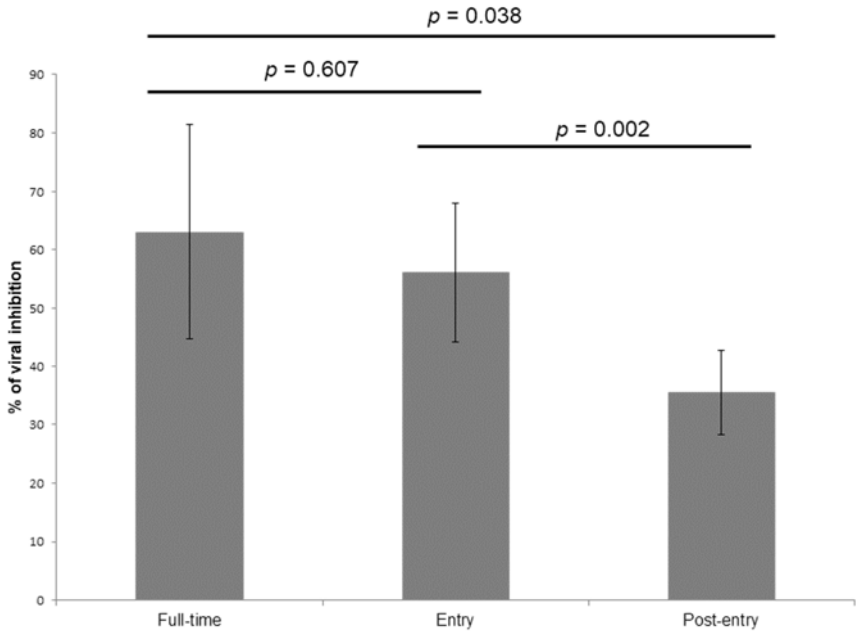


Figure 2. Antiviral activities of doxycycline at 5 µM against the SARS-CoV-2 IHUMI-003 strain in vitro. For full-time treatment, Vero E6 cells were pre-treated with doxycycline for 4 h and virus was then added for 48 h. For "entry" treatment, doxycycline was added to Vero E6 cells 4 h before viral infection and the virus-doxycycline mixture was replaced with fresh medium after 2 h post infection and was maintained for 46 h. For "post-entry" treatment, doxycycline was added 2 h post infection and was maintained for 46 h. Error bars represent the standard deviation of 10 experiments.

3. Discussion

The in vitro activity of doxycycline against the SARS-CoV-2 (EC_{50} = 4.5 µM) was consistent with those reported with hydroxychloroquine [27,29–31], with antimalarial drugs like chloroquine [27–29,32], amodiaquine [29,31,32], ferroquine [29], or mefloquine [29,31,33], antiviral agents like remdesivir [28,32,34] or lopinavir [32], and macrolides like azithromycin [34,35].

A daily oral uptake of 100 mg or 200 mg of doxycycline in healthy volunteers led to a C_{max} (maximum blood concentration) value of 1.7 and 5 µg/mL [36,37]. An intravenous doxycycline dose of 100 mg showed a C_{max} of 2.6 µg/mL [38]. The ratios C_{max}/EC_{50} and C_{max}/EC_{90} for doxycycline in plasma ranged from 0.75 to 2.21 and 0.07 to 0.41, respectively. The ratios C_{max}/EC_{50} and C_{max}/EC_{90} in plasma seem low to reach effective concentrations to inhibit SARS-CoV-2 in humans. However,

FOI 26-3132 - Document 5

in lungs, doxycycline were 2- to 4-fold higher than in plasma [39,40]. A daily oral uptake of 100 or 200 mg of doxycycline led to a C_{max} value from 3.4 to 20 μg/g of lungs. The ratios C_{max}/EC_{50} and C_{max}/EC_{90} for doxycycline in lungs ranged from 1.5 to 8.8 and 0.15 to 1.6, respectively.

Doxycycline interacted at both entry and post-entry stages of the SARS-CoV-2 infection in Vero E6 cells. Doxycycline inhibited in vitro both dengue virus replication by interaction with the dengue virus serine protease (DENV2 NS2B-NS3pro) and dengue viral entry by inhibition of the E2 envelope glycoprotein involved in virus entry [20,22]. Doxycycline interfered particularly with the adsorption and entry of the Chikungunya virus by interaction with the E2 envelope glycoprotein, but also inhibited viral replication in a lower manner [23]. However, doxycycline had no effect in vitro on the adsorption and entry of the vesicular stomatitis virus (VSV) and inhibited only viral replication [19]. Moreover, doxycycline enhanced the adsorption of the porcine reproductive and respiratory syndrome virus (PRRSV) and inhibited its replication after viral entry [25]. The inhibition of both entry and viral replication after SARS-CoV-2 entry is consistent with the results from combinatorial computational approaches. Docking analysis showed that doxycycline could strongly bind the spike protein (S) of SARS-CoV-2 [41]. The spike viral protein of SARS-CoV-2 used the ACE-2 receptor for entry [42]. Therefore, blocking these interactions offers a potential target for drug development. Moreover, doxycycline and more generally tetracyclines could bind the main protease (M^{pro}) of SARS-CoV-2 [43,44]. This protein, also called 3C-like protease, is essential to conduct the replication cycle of SARS-CoV-2 by leading to the formation of non-structural proteins (NSPs) [45,46]. Moreover, Alexpandi et al. showed that doxycycline inhibited the RNA-directed 5′-3′ polymerase activity of SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) (Alexpandi et al., personal data), a key enzyme for the replication of the SARS-CoV-2 [47], like remdesivir [48,49].

However, these results must be taken with caution regarding the potential use of antimalarial drugs in SARS-CoV-2-infected patients: it is difficult to translate in vitro study results into actual clinical treatment in patients. For instance, experts agree on the in vitro activity of chloroquine or hydroxychloroquine against SARS-CoV-2 but disagree on the hydroxychloroquine efficacy in COVID-19 treatments, which remains controversial [50,51]. In vivo evaluation in animal experimental models is required to confirm the antiviral effects of doxycycline on SARS-CoV-2.

Besides its antiviral activity, doxycycline has anti-inflammatory effects by decreasing the expression of various pro-inflammatory cytokines including interleukins 1 (IL-1), 6 (IL-6), and 8 (Il-8) and tumor necrosis factor-alpha (TNF-α) by macrophages [17,52] and chemokines including monocyte chemotactic protein 1, macrophage inflammatory protein 1α and 1β [53]. The immunomodulatory activity of doxycycline improved survival of septic mice with pulmonary inflammation [54]. Moreover, doxycycline is effective for the treatment of several chronic inflammatory airway diseases, including acute respiratory distress syndrome [55]. Doxycycline was the more effective tetracycline in the reduction of IL-6 and TNF-α in patients with dengue fever [56]. Doxycycline ameliorated pulmonary inflammation in a murine polymicrobial sepsis model by decreasing levels of IL-1β, IL-6 and TNFα in plasma and lungs [54]. Doxycycline improved the lung function and quality of life in chronic obstructive pulmonary disease (COPD) [57]. Levels of IL-6, IL-8 and TNFα were reduced in doxycycline-treated COPD patients. Moreover, doxycycline reduced neuroinflammation and preserved oxidative balance in traumatic brain injury-induced cognitive/motor impairments in rats due to its anti-inflammatory and free scavenging mechanisms [58]. Doxycycline could be a potential partner of COVID-19 therapies due to its anti-inflammatory effects.

Moreover, doxycycline is a bacteriostatic antibiotic that is active against the bacterial causes of community-acquired pneumonia, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and atypical pathogens such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella spp* [59]. Doxycycline is used commonly for lower respiratory tract infection [60]. The use of doxycycline as antimalarial prophylaxis is associated with a reduced risk of Influenza-like illness among travelers [61]. Even without antiviral effects, doxycycline could be a potential partner of COVID-19 therapies due to anti-inflammatory effects and antibiotic effects that could prevent co-infections and superinfections.

FOI 26-3132 - Document 5

Early treatment with doxycycline of 100 mg a day for 7 days (per os or intravenous) in 89 high-risk patients with moderate to severe COVID-19 infections was associated with early clinical recovery, decreased hospitalization and reduced mortality [62]. Another observational study on 54 high-risk patients in 3 long term care facilities in New York revealed that the use of a combination of 100 mg of doxycycline twice a day for 7 days and 400 mg of hydroxychloroquine twice a day on the first day and daily for the next 6 days was associated with a decrease in transfer to hospital and reduced mortality [63]. Results were compared and analyzed against the data observed in a long term care facility in Washington. However, these results must be confirmed by rigorous studies. Many clinical trials on doxycycline alone or in combination to treat COVID-19 are in progress [64–66].

4. Materials and Methods

4.1. Agent, Virus, and Cells

Stock solution of doxycycline hyclate (Sigma, Saint Louis, MO, USA) was prepared in methanol and diluted in Minimum Essential Media (MEM, Gibco, ThermoFischer, Waltham, MA, USA) in order to have 7 final concentrations ranging from 0.1 µM to 100 µM. Chloroquine diphosphate (Sigma) was used as comparator. The clinically isolated SARS-CoV-2 strain (IHUMI-3) [4] was maintained in production in Vero E6 cells (American type culture collection ATCC® CRL-1586™) in MEM with 4% of fetal bovine serum and 1% glutamine (complete medium).

4.2. Cytotoxicity Assay

In vitro cell viability evaluation on the VERO E6 cell line was performed according to the method described by Mosmann with slight modifications [67]. Briefly, 10^5 cells in 200 µL of complete medium were added to each well of 96-well plates and incubated at 37 °C in a humidified 5% CO_2. After 24 h incubation, 25 µL of complete medium and 25 µL of each concentration of methylene blue, hydroxychloroquine or azithromycin were added and the plates were incubated for 48 h at 37 °C. After removal of the surpernatant, 100 µL of MTT (3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2*H*-tetrazolium bromide, Sigma Aldrich, St Quentin Fallavier, France) solution (0.5 mg/mL in MEM without FBS) was then added to each well. Cells were incubated for 2 h at 37 °C. After incubation, the MTT solution was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Then, plates were shaken at 700 rpm for 10 min at 37 °C. The absorbance was measured at 570 nm using a TECAN Infinite F200 Microplate Reader. DMSO was used as blank. The 50% cytotoxicity concentration (CC_{50}) was calculated with the inhibitory sigmoid E_{max} model, which estimated the CC_{50} through nonlinear regression by using a standard function of the R software (ICEstimator version 1.2) [68]. CC_{50} value resulted in the mean of 5 different experimentations.

4.3. Antiviral Activity Assay

Briefly, 96-well plates were prepared with 5.10^5 cells/mL of Vero E6 (200 µL per well), as previously described. Doxycycline and chloroquine concentrations were added 4 h before infection. Vero E Cells were infected with IHUMI-3 strain at an MOI of 0.25. After 48 h post-infection, the replication was estimated by RT-PCR using the Superscrit III platinum one step with Rox kit (Invitrogene) after extraction with the BIoExtract SuperBall kit (Biosellal, Dardilly, France). The primers used were previously described [69]. The percentage of inhibition of SARS-CoV-2 replication was estimated for each drug concentration as following: (mean $CT_{\text{drug concentration}}$ − mean $CT_{\text{control 0\%}}$)/(mean $CT_{\text{control 100\%}}$ − mean $CT_{\text{control 0\%}}$) × 100. EC_{50} (median effective concentration) and EC_{90} (90% effective concentration) were calculated with the inhibitory sigmoid E_{max} model, which estimated the EC_{50} and EC_{90} through nonlinear regression by using a standard function of the R software (ICEstimator version 1.2). EC_{50} and EC_{90} values resulted in the mean of 10 different experimentations.

FOI 26-3132 - Document 5

4.4. Determination of the Inhibition Stage

Determining in vitro at what stage doxycycline is acting against the SARS-CoV-2 IHUMI-003 strain was assessed at a concentration of at 5 μM. For "full-time treatment", Vero E6 cells were pre-treated with doxycycline for 4 h and virus was then added for 48 h. For "entry" treatment, doxycycline was added to Vero E6 cells 4 h before viral infection and the virus-doxycycline mixture was replaced with fresh medium after 2 h post infection and was maintained for 46 h. For "post-entry" treatment, doxycycline was added 2 h after post infection and was maintained for 46 h. The percentage of inhibition of SARS-CoV-2 replication by 5 μM of doxycycline was estimated for each drug concentration as following: (mean $CT_{\text{drug concentration}}$ − mean $CT_{\text{control 0\%}}$)/(mean $CT_{\text{control 100\%}}$ − mean $CT_{\text{control 0\%}}$) × 100. The result was the mean of 10 different experiments.

4.5. Data Analysis and Interpretation

Results were estimated as a mean and standard deviation of 5 to 10 experiments. Selectivity index (SI) as a ratio of CC_{50}/EC_{50} was estimated for doxycycline. The expected maximum blood concentration (C_{max}) was estimated from the literature for doxycycline at doses commonly administered in oral or intravenous treatment. The ratios C_{max}/EC_{50} and C_{max}/EC_{90} were estimated to find out if the effective concentration in plasma to cure SARS-CoV-2 is achievable in humans. The ratios C_{lung}/EC_{50} and C_{lung}/EC_{90} were estimated from the data on doxycycline accumulation into the lung.

5. Conclusions

In conclusion, doxycycline showed a high in vitro antiviral effective activity against SARS-CoV-2 with IC_{50} (4.5 μM) compatible with oral uptake and intravenous administrations. Doxycycline interacted both on SARS-CoV-2 entry and in replication after virus entry. Besides its in vitro antiviral activity against SARS-CoV-2, doxycycline has anti-inflammatory effects by decreasing the expression of various pro-inflammatory cytokines and could prevent co-infections and superinfections due to broad-spectrum antimicrobial activity. Therefore, doxycycline could be a potential partner of COVID-19 therapies. However, these results must be taken with caution regarding their potential use in SARS-CoV-2-infected patients: it is difficult to translate in vitro study results into actual clinical treatment in patients. In vivo evaluation in animal experimental models is required to confirm the antiviral effects of doxycycline on SARS-CoV-2 and more trials of high-risk patients with moderate to severe COVID-19 infections must be initiated.

Author Contributions: Conceptualization, M.G., B.L.S. and B.P.; validation, M.G., S.H., H.B. and B.P.; formal analysis, B.P.; investigation, M.G., J.A., O.D., M.B., J.M. and I.F.; resources, S.H., P.J., M.L.B., I.D. and C.R.; writing—original draft preparation, M.G., B.L.S. and B.P.; writing—review and editing, J.A., and S.H.; supervision, B.L.S. and B.P.; project administration, B.P.; funding acquisition, B.L.S. and B.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Agency, program "Investissement d'avenir"grant number ANR-10-IAHU-03" and Institut Hospitalo-Universiare (IHU) Méditerranée Infection grant number COVID-19. Manon Boxberger received e PhD grant supported by L'Occitane Society.

Conflicts of Interest: The authors have no conflict of interest to declare. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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Sample Availability: Samples of the compounds are not available from the authors.

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FOI 26-3132 - Document 5

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Tissue Distribution of Doxycycline in Animal Models of Tuberculosis

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ABSTRACT Doxycycline, an FDA-approved tetracycline, is used in tuberculosis *in vivo* models for the temporal control of mycobacterial gene expression. In these models, animals are infected with recombinant *Mycobacterium tuberculosis* carrying genes of interest under transcriptional control of the doxycycline-responsive TetR-*tetO* unit. To minimize fluctuations of plasma levels, doxycycline is usually administered in the diet. However, tissue penetration studies to identify the minimum doxycycline content in food achieving complete repression of TetR-controlled genes in tuberculosis (TB)-infected organs and lesions have not been conducted. Here, we first determined the tetracycline concentrations required to achieve silencing of *M. tuberculosis* target genes *in vitro*. Next, we measured doxycycline concentrations in plasma, major organs, and lung lesions in TB-infected mice and rabbits and compared these values to silencing concentrations measured *in vitro*. We found that 2,000 ppm doxycycline supplemented in mouse and rabbit feed is sufficient to reach target concentrations in TB lesions. In rabbit chow, the calcium content had to be reduced 5-fold to minimize chelation of doxycycline and deliver adequate oral bioavailability. Clearance kinetics from major organs and lung lesions revealed that doxycycline levels fall below concentrations that repress *tet* promoters within 7 to 14 days after doxycycline is removed from the diet. In summary, we have shown that 2,000 ppm doxycycline supplemented in standard mouse diet and in low-calcium rabbit diet delivers concentrations adequate to achieve full repression of *tet* promoters in infected tissues of mice and rabbits.

KEYWORDS doxycycline, TetR, tuberculosis, animal models, tissue penetration, genetic regulation *in vivo*

Transcriptional regulators derived from the tetracycline repressor (TetR) of *Escherichia coli* have been exploited for more than 2 decades to chemically regulate gene expression with tetracyclines in eukaryotic cells (1–3). The specificity of the Tet repressor-operator-effector interaction and the favorable *in vivo* pharmacokinetics of tetracyclines make this regulatory system well suited for the temporal control of gene expression *in vivo*, such as in transgenic animals (4) and animals infected with recombinant pathogenic bacteria carrying genes of interest under the TetR-*tetO* transcriptional control unit (5–7).

Promoters that can be controlled by TetR and tetracyclines were initially developed *in vitro* in Gram-positive and Gram-negative bacteria (8). More recently, such systems have also been developed for medically relevant acid-fast bacteria, including *M. tuberculosis* (9, 10). Owing to its excellent oral bioavailability and pharmacokinetic properties in mice and its relative lack of activity against *M. tuberculosis* with an MIC of 8 to 16 μg/ml (11), doxycycline (DOX) has frequently been used in tuberculosis (TB) mouse

Citation Gengenbacher M, Zimmerman MD, Sarathy JP, Kaya F, Wang H, Mina M, Carter C, Hossen MA, Su H, Trujillo C, Ehrt S, Schnappinger D, Dartois V. 2020. Tissue distribution of doxycycline in animal models of tuberculosis. Antimicrob Agents Chemother 64:e02479-19. https://doi.org/10.1128/AAC.02479-19.

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Received 11 December 2019
Returned for modification 24 January 2020
Accepted 6 February 2020
Accepted manuscript posted online 10 February 2020
Published 21 April 2020

models to induce or repress *M. tuberculosis* genes (12–15). In general, the affinity of DOX for TetR is several orders of magnitude higher than its affinity for the ribosome, providing a safe range within which genetic regulation can be achieved without affecting bacterial growth (16).

To regulate *M. tuberculosis* gene expression during infection, DOX is administered either in the drinking water at up to 2 mg/ml or in commercially available modified diets at 200 to 2,000 ppm, where it was reported to be stable for at least 7 days and 6 months, respectively (17, 18). Supplementation of DOX in mouse feed is considered the most convenient method to achieve adequate and consistent plasma levels (18).

Since DOX is commonly used as a broad-spectrum antibiotic in veterinary medicine and the food industry, tissue distribution studies have been conducted in animals such as horses and broiler chickens to assess its penetration into various organs and cell types. DOX shows favorable tissue partitioning and equilibration of free drug concentrations (with a free fraction in plasma of 20%) between plasma and interstitial fluid at steady state. Accumulation in specific cell types, such as polymorphonuclear leukocytes (19) and fibroblasts (20, 21), has been observed. However, no tissue penetration study has been conducted to determine whether adequate DOX levels are achieved in TB-infected organs and immune cell types in which the pathogen resides.

This work was undertaken to (i) identify the range of DOX concentrations that achieve full repression of Tet-OFF promoters *in vitro*, (ii) measure DOX concentrations in major organs and in lung lesions in TB-infected mice and rabbits in order to determine the lowest DOX feed content required to achieve the target concentrations identified *in vitro*, and (iii) explore the clearance kinetics of DOX from lung tissue and lesions to determine the number of days required for DOX levels to fall below concentrations sufficient to induce or repress *tet* promoters after DOX is removed from the diet.

RESULTS

Concentrations required for control of Tet-OFF constructs by tetracyclines *in vitro*. Anhydrotetracycline (ATc) is conventionally used as the *in vitro* inducer or repressor of TetR-*tetO* constructs due to its lack of growth inhibitory activity against *M. tuberculosis*. DOX, on the other hand, is the preferred transcriptional regulator *in vivo* because it is orally bioavailable and inhibits *M. tuberculosis* growth only at high concentrations (11). To determine the concentrations of ATc and DOX required to achieve complete silencing of target genes in *M. tuberculosis in vitro*, we resorted to genetically engineered strains in which a dual-control switch combining repression of transcription and controlled proteolysis had been chromosomally integrated to silence gene and protein activities (14, 22–24). Three *M. tuberculosis* strains, each with a dual-control switch targeting either *fum*, *rho*, or *trxB2* (three essential *M. tuberculosis* genes), were exposed to increasing concentrations of ATc or DOX. ATc concentrations ranging from 1 to 2 μg/ml were required to achieve 90% growth inhibition resulting from effective silencing in all three strains (Fig. 1A). When DOX was used as the TetR ligand *in vitro*, 50% growth inhibition was achieved at 80, 120, and 200 ng/ml for *trxB2*, *rho*, and *fum*, respectively; 90% growth inhibition was achieved between 230 and 350 ng/ml (Fig. 1B). Thus, lower DOX than ATc concentrations are required to control TetR-*tetO* constructs in *M. tuberculosis*. To ensure that these concentrations do not interfere with the growth of wild-type *M. tuberculosis* and to confirm published MICs, full dose-response curves were obtained with *M. tuberculosis* H37Rv exposed to both tetracyclines. *M. tuberculosis* growth remained unaffected by ATc up to the highest concentration tested (4 μg/ml), while DOX achieved approximately 30% growth inhibition at 4 μg/ml (Fig. 1C), consistent with the published MICs of 8 to 16 μg/ml (11). These results indicate that the concentrations required to achieve maximum control of TetR-*tetO* constructs do not affect growth of *M. tuberculosis in vitro*.

Pharmacokinetics and tissue distribution of DOX in mice. To compare the DOX concentrations achieved in mice relative to DOX MICs and concentrations required to regulate TetR-controlled promoters, we first measured DOX concentrations in the

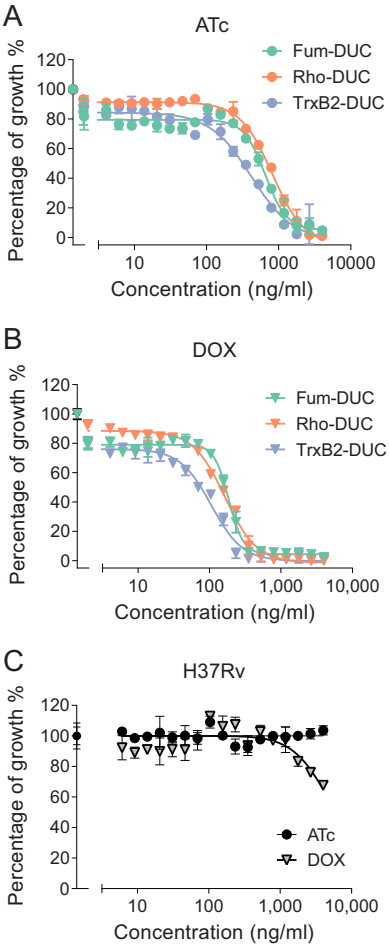


FIG 1 Effect of tetracyclines on *in vitro* growth of *M. tuberculosis* H37Rv harboring essential genes under TetR control compared to wild type. (A and B) Growth inhibitory dose response of anhydrotetracycline (ATc) and doxycycline (DOX) in *M. tuberculosis* strains that harbor a TetR-dependent dual-control genetic switch (14) driving expression of Fum (fumarate hydratase, green symbols), Rho (transcription termination factor, orange symbols), or TrxB2 (thioredoxin reductase, blue symbols). (C) Growth inhibitory dose response of ATc and DOX against wild-type *M. tuberculosis* H37Rv.

plasma of TB-infected mice receiving 2,000 ppm DOX in the diet. Plasma was collected on days 1, 2, 3, and 7 following the switch from DOX-free to DOX-medicated chow, 3 weeks postinfection with *M. tuberculosis* strain H37Rv. Plasma levels of DOX ranged from 300 to 1,190 ng/ml (average, 637 ng/ml; standard deviation [SD], 411 ng/ml) on day 1 and reached an average of 1,081 ng/ml (SD, 386 ng/ml; range, 757 to 1,850 ng/ml) on day 7 (Fig. 2A), thus achieving the concentrations required to inhibit 90% of the growth of strains harboring representative Tet-OFF-controlled essential genes *in vitro* (Fig. 1B). To assess potential lot-to-lot variability in DOX chow, groups of 3 mice were fed three different lots, and plasma DOX concentrations were measured in serial blood samples from 0 to 5 h on day 1 (see Fig. S1 in the supplemental material), showing consistent plasma levels across lots. Actual DOX concentrations were measured in each lot and found to be within 25% of the target content of 2,000 ppm (Table S1).

To quantify penetration into tissues, we measured DOX concentrations in all major organs in the TB-infected mice that had received DOX-medicated chow for 7 days (Fig. 2B), showing favorable penetration in all organs except for brain tissue. In infected lung, spleen, and lymph node homogenates, total DOX concentrations were 2- to 4-fold higher than in plasma, thus exceeding levels required to achieve 90% growth inhibition, while not reaching the MIC of 8 to 16 μg/ml (Fig. 2B). To determine the time required for DOX to clear plasma and infected organs below concentrations sufficient

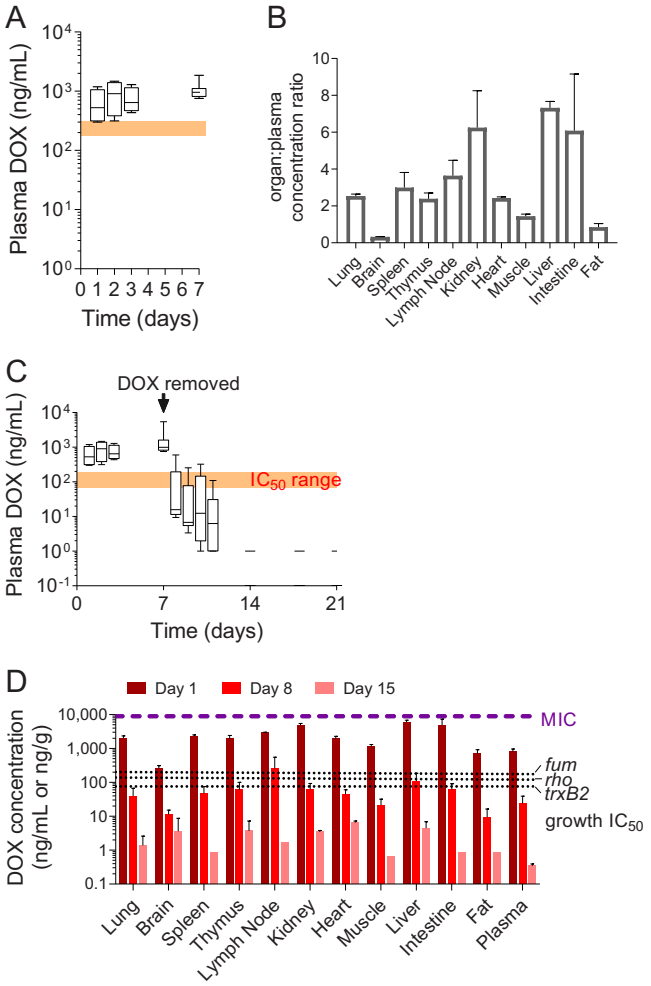


FIG 2 Plasma and tissue pharmacokinetics of DOX in mice receiving DOX-supplemented diet. (A) Box and whisker plot of DOX plasma concentrations in mice receiving 2,000 ppm DOX in standard diet for 7 days. Mean values (bar), minimum and maximum (box) and standard deviations (error bars) are shown for $n = 4$ to 11 mice per group. The orange shaded window indicates the range of *in vitro* DOX concentration that inhibits 90% of bacterial growth (IC_{90}) for the three reporter strains shown in Fig. 1. (B) DOX concentrations in mouse organs relative to plasma after 7 days on the DOX diet. Mean values and standard deviations (error bars) are shown ($n = 3$). (C) Kinetics of DOX clearance in plasma following removal of DOX from the diet for 14 days. Mean values (bar) and minimum and maximum (box) and standard deviations (error bars) are shown ($n = 3$ to 12). The orange shaded window indicates the range of *in vitro* DOX concentrations that inhibit 50% of bacterial growth (IC_{50}) for the three reporter strains shown in Fig. 1. (D) Kinetics of DOX clearance from major organs of TB-infected mice 7 and 14 days after removal of DOX from the diet. Day 1 corresponds to DOX levels in mice that had received a DOX-supplemented diet for 1 week, after which it was replaced by a DOX-free diet for the duration of the experiment. The purple dotted line indicates the lower boundary of published MIC ranges against wild-type *M. tuberculosis* (11), and the black dotted lines show individual DOX IC_{50}s for the three reporter strains (Fig. 1). Mean values and standard deviations (error bars) are shown ($n = 3$).

to induce or repress Tet-ON and Tet-OFF promoters, mice were switched to a DOX-free diet after 7 days on DOX chow, and DOX concentrations in plasma were measured at 1, 2, 3, 4, 7, 11, and 14 days post-DOX removal (Fig. 2C). To determine how this translated within tissue, we quantified DOX in homogenized organs from 3 mice per group at 1, 8, and 15 days post-DOX removal, showing that concentrations fell below the levels required to achieve 50% growth inhibition in all organs except lymph nodes within 8 days post-DOX removal (Fig. 2D).

Dose finding pharmacokinetics of DOX in rabbits. The rabbit model of TB disease is well suited to assess the essentiality and vulnerability of bacterial targets in mature cellular and necrotic lesions resembling those found in human TB (25, 26). However,

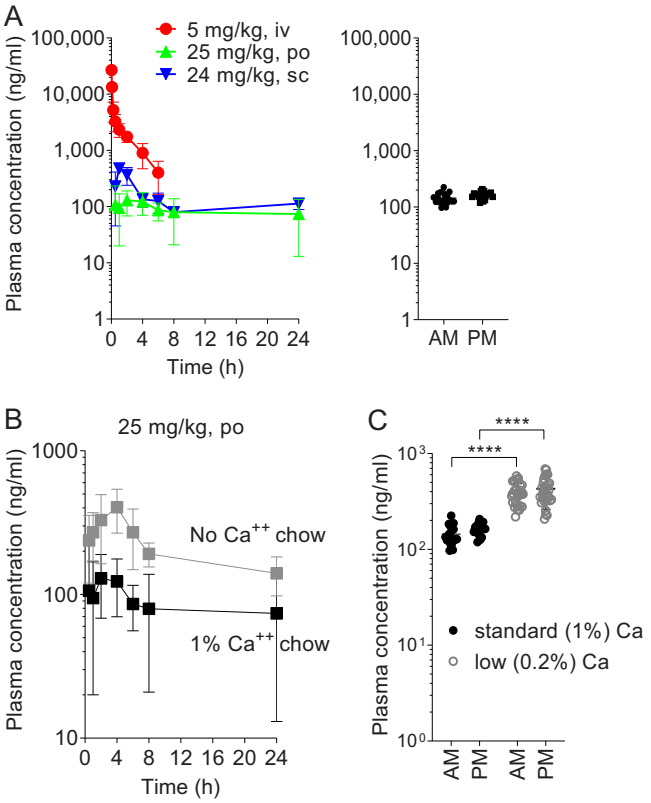


FIG 3 Plasma and tissue pharmacokinetics of DOX in rabbits receiving DOX-supplemented chow. (A) Left panel: DOX concentration-time profile in rabbit plasma following a single DOX dose administered intravenously (i.v.), by oral gavage (p.o.), and subcutaneously (s.c.), as indicated. Right panel: DOX plasma concentrations in rabbits receiving 2,000 ppm DOX in standard chow for 7 days. AM, immediately prior to receiving daily DOX-supplemented feed in the morning; PM, 6 h after receiving DOX feed. (B) Effect of dietary calcium removal on the plasma pharmacokinetics of DOX in rabbits following a single oral dose of 25 mg/kg. (C) Effect of dietary calcium lowering (0.2% total calcium content, or 5-fold lower than in standard chow) on DOX plasma levels in rabbits that received 2,000 ppm DOX in chow for 5 to 7 days. AM and PM time points are as described for right panel A. Data are represented as scatter dot plots of individual animal values and are pooled from two separate experiments with 4 and 6 rabbits, respectively (horizontal bar indicates mean, $n = 20$ to 34 blood samples from 10 rabbits).

DOX pharmacokinetics (PK) and distribution in pulmonary lesions have not been characterized in rabbits. Preliminary PK studies in rabbits that received a modified diet with 200 and 400 ppm DOX showed unexpectedly low plasma levels (Fig. S2A). When the DOX content of the rabbit diet was increased to 2,000 ppm (corresponding approximately to 100 mg/kg), plasma levels increased proportionally, but remained lower than in mice, at 100 to 200 ng/ml throughout the 10-day PK study (Fig. 3A).

To determine whether poor oral bioavailability or species-specific metabolism was the reason for the low DOX concentration in rabbits, we dosed uninfected rabbits with DOX via the intravenous (i.v.), oral (p.o.), and subcutaneous (s.c.) routes. Following bolus i.v. dosing, a biphasic concentration-time profile was observed in plasma, with a distribution and elimination phase, consistent with a two-compartment PK model (Fig. 3A, Table 1). The volume of distribution at steady state (steady-state volume of distribution [V_{dss}] or volume of distribution [V_d] area) was high, reaching a maximum of approximately 1 liter/kg, corresponding to total body volume and similar to the V_d measured in domestic rabbits (27) and in pigs (28). The clearance was low at 26 ml/min or 15% of liver blood flow (Table 1). The i.v. concentration time profile and PK parameters were consistent with published data that used a bioassay for DOX quantitation following i.v. injection in domestic rabbits (27). DOX administration by oral gavage resulted in 3.2% bioavailability (Fig. 3A, Table 1), indicating that poor absorption rather than fast metabolism may partially account for the low oral bioavailability.

FOI 26-3132 - Document 5

TABLE 1 Pharmacokinetic parameters of DOX in rabbits[a]

Route	Dose (mg/kg)	AUC_{0-24} (ng/h/ml)	C_{ave} (ng/ml)	Elim $T_{1/2}$ (h)	k_{el} (h^{-1}) (liters/kg)	V_{dss}/*V* area (liters/kg)[b]	CL (ml/min)	F (%)
Intravenous bolus	5	12,478 (4,058)	NA	1.95 (0.49)	0.35 (0.08)	1,056/1,138	26.2 (5.6)	NA
Oral gavage	25	2,046 (973)	85 (40)					3.2
Subcutaneous	24	3,175 (356)	132 (15)					5.2
Oral gavage (standard 1% calcium chow)	25	2,317 (275)	97 (11)					3.6
Oral gavage (calcium-free chow)	25	5,014 (950)	209 (40)					7.9
Oral gavage (TB-infected)	20	360	15					0.7

[a]AUC_{0-24}, area under the concentration-time curve; Elim $T_{1/2}$, elimination half-life; C_{ave}, average plasma concentration over 24 h; k_{el}, elimination rate constant; CL, clearance; F, bioavailability.
[b]V_{dss} = dose · [(A/alpha^2 + B/beta^2)/AUC^2]; *V* area = dose/beta kel · AUC (45).

To determine whether pharmaceutical grade formulation might improve oral absorption, we dosed rabbits with DOX tablets at approximately 15 and 30 mg/kg, which did not result in detectable exposure improvement (Fig. S2B). Lastly, subcutaneous injection resulted in a 1.5-fold increase in the area under the concentration-time curve (AUC) and average concentration throughout the dosing interval (Fig. 3A, Table 1).

Calcium found in dairy products interferes with tetracycline oral absorption because it forms insoluble chelates resulting in decreased bioavailability (29). While most mammals tightly regulate blood calcium, rabbits absorb nearly all dietary calcium and excrete the excess through the kidneys. This results in a surge of blood calcium following food intake (30), proportional to calcium levels in the diet. We therefore hypothesized that DOX absorption and plasma concentrations may increase in rabbits receiving a calcium-free or low-calcium diet. Rabbits were fed a calcium-free or 1% calcium diet for a minimum of 2 days, at which point they received a single 25-mg/kg dose of DOX via oral gavage. As hypothesized, exposure (AUC) after a single dose was twice as high in rabbits on a calcium-free diet (Fig. 3B, Table 1).

To determine whether a similar improvement in DOX exposure would be seen when DOX is provided in the chow, under dietary conditions compatible with long-term experiments (i.e., not calcium-free), we placed rabbits on either standard (1% calcium) chow with DOX at 2,000 ppm or 0.2% calcium chow with DOX at 2,000 ppm, as described in Materials and Methods. Blood samples were collected twice a day (a.m. and p.m.) after 5 to 7 days on DOX chow as indicated. The respective mean a.m. and p.m. plasma DOX concentrations were 144 and 163 ng/ml in the standard-calcium group and 385 and 429 ng/ml in the low-calcium group (Fig. 3C), showing a 2 to 3-fold increase in the low-calcium group and reinforcing the trend observed when DOX was received as a single oral dose. Having optimized constant DOX exposure when provided in the diet, we next studied its distribution in the cellular and necrotic compartments of mature rabbit lesions in order to determine the DOX concentrations to which *M. tuberculosis* bacilli are exposed in tissues.

***In vitro* lesion pharmacokinetics.** Previous work by our group indicated that drug distribution and partitioning within lesions is a function of uptake into macrophages and binding to caseum macromolecules (31, 32). To predict the penetration of DOX in cellular lesions and its behavior at the interface between the cellular rim and necrotic core of TB lesions, we measured DOX uptake into macrophages and caseum binding. DOX uptake was quantified in THP-1 macrophages, where it reached an intracellular to extracellular concentration ratio of 4.1 (SD, 0.6). This favorable cellular uptake predicted good penetration into cellular lesions. The average unbound drug fraction in caseum ($f_{u\text{-caseum}}$) was moderate to high at 12 to 14% (SD 3%) of total drug. Together with the moderate uptake in macrophages, the DOX fraction unbound predicted favorable partitioning at the cellular/caseum interface and good passive diffusion through nonvascularized caseum. Taken together, these results suggest that DOX should reach higher concentrations in both cellular and necrotic lesions than plasma.

Distribution of DOX in cellular and necrotic lesion compartments. Uninvolved lung, cellular, and necrotic lesions were dissected from the lungs of TB-infected rabbits

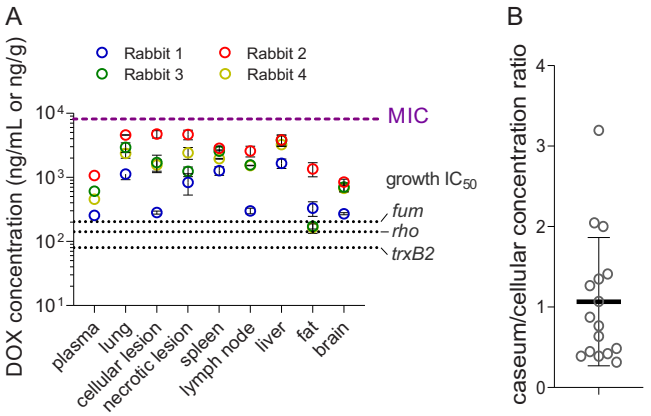


FIG 4 DOX penetration in major organs, lung lesions, and necrotic caseum of TB-infected rabbits. (A) DOX concentrations in plasma, uninvolved lung tissue, lung lesions, and major organs in 4 rabbits receiving a low-calcium 2,000 ppm DOX diet for 7 days. Mean values and standard deviations (error bars) are shown ($n = 3$). (B) Scatter dot plots of caseum-to-cellular DOX concentration ratios in necrotic granulomas collected from TB-infected lungs. Ratios obtained in 10 lesions from 5 infected rabbits are shown.

receiving 2,000 ppm DOX in low-calcium (20% of standard or 0.2% final) chow for 7 days. DOX was also quantified in spleen, lymph nodes, liver, fat, and brain tissue. We found higher DOX levels in cellular and necrotic lesions than in plasma (Fig. 4A), indicating that DOX accumulates in infected tissues relative to plasma, as predicted by the macrophage uptake assay and consistent with the reported accumulation in polymorphonuclear leukocytes (19). Favorable penetration was also measured in spleen, liver, and lymph nodes. All tissue concentrations were above the levels required to achieve 90% growth control, with the exception of one animal with borderline DOX concentrations in some of the tissues. These lower concentrations were explained by low food consumption (Table S2). DOX levels were below the *M. tuberculosis* MIC in all tissues (Fig. 4A). To quantify the partitioning of DOX at the cellular/caseum interface, we used laser-capture microdissection in thin sections of large necrotic lesions (33) from the same rabbits and found caseum-to-cellular DOX concentration ratios ranging from 0.3 to 3.2 with a mean value of around 1.0 (Fig. 4B). Overall, we found (i) higher DOX concentrations in fully cellular lesions than in plasma in line with the macrophage uptake data and (ii) comparable concentrations in caseum and cellular rims, confirming the favorable passive diffusion of DOX through avascular caseum predicted by the caseum binding assay. Collectively, these results indicate that in rabbits on low-calcium chow supplemented with DOX at 2,000 ppm, DOX concentrations are sufficient to achieve complete repression of Tet-OFF genetic constructs but not high enough to affect growth and viability of wild-type *M. tuberculosis*.

DISCUSSION

We have shown that 2,000 ppm DOX supplemented in a standard mouse diet and in a low-calcium rabbit diet deliver DOX concentrations adequate to achieve full repression of representative Tet-OFF promoters in infected tissues of both mice and rabbits. These concentrations remain below the DOX MIC in all tissues and are thus likely to primarily affect *M. tuberculosis* growth and viability by regulation of genes under the control of TetR. The clearance kinetics of DOX from lung lesions and other infected organs indicate that DOX levels fall below concentrations sufficient to repress *tet* promoters within 7 days after DOX is removed from the diet.

DOX bioavailability is species specific and subject to food effect. It is very poor in rats (34) and moderate to low (20%) in pigs (28) but almost complete in calves (35) and humans (36). In nonfasted rabbits that received 150 mg/kg tetracycline by oral gavage (27), bioavailability was estimated at 4% (Data Set S1), in line with the present findings. Interestingly, DOX exposure was also very low in rabbits that received subcutaneous

injections as part of this work, indicating that low bioavailability is not exclusively due to poor oral absorption. Tetracyclines are known to localize in bones and teeth, where 99% of the body calcium is found and where they form calcium-bound depots. They also accumulate in liver and spleen, where they are more highly bound than in plasma (http://www.cyto.purdue.edu/cdroms/cyto2/17/chmrx/tetra.htm). Such trapping in selected tissues could contribute to species-specific plasma levels. In early studies, the distribution of DOX in tissues was described in rats (37) and in patients undergoing biopsies during surgery (38). DOX concentrations were measured in plasma/serum and in major organs using a bioassay and indicated lung-to-serum concentration ratios between 2 and 3, consistent with our findings. Penetration was highest in kidneys and intestines and lowest in fat tissue (37, 38), again in line with our results (Fig. 2B).

Rabbits possess a unique calcium metabolism. Most mammals regulate blood calcium to maintain levels within a narrow range by controlling the absorption of calcium from the diet according to need. Rabbits have adopted the different strategy of absorbing nearly all dietary calcium ingested and renally excreting the excess. As a result, blood concentrations transiently reflect calcium levels in the diet, and calcium carbonate is excreted in large amounts in the urine (30). It has been determined that 0.2% calcium in rabbit chow is sufficient to sustain normal growth and weight gain (30), whereas standard diets contain 1% calcium. Reducing calcium content to 0.2% in rabbit chow supplemented with 2,000 ppm DOX led to improved oral bioavailability and at least 2-fold higher plasma levels throughout the day. DOX concentrations in all relevant organs, in lung lesions, and in plasma were higher than the minimum required to regulate *tet* promoters and did not reach the lower threshold of published MICs in broth (8 to 16 mg/liter [11]). Similar MICs have been measured against intracellular *M. tuberculosis* in macrophages, and bactericidal activity was observed at 40 mg/liter (39). DOX showed favorable partitioning into the necrotic foci of caseous lesions, with an average caseum/cellular concentration ratio of 1/1. Since caseum *M. tuberculosis* is nonreplicating and overall phenotypically resistant to most TB drugs, DOX is not expected to affect *M. tuberculosis* viability in this lesion compartment. Given the similar penetration of DOX in uninvolved lung, cellular, and caseous lesions (Fig. 4A), partial lesion resolution over time due to effective gene silencing by DOX and reduction of bacterial burden is unlikely to impact DOX penetration. Slow tissue clearance has been reported for DOX (40), consistent with our observation that levels fall below effective concentrations after approximately 7 days on a DOX-free diet. These findings have implications as to the kinetics of *tet* regulation following DOX removal and should be taken into consideration when interpreting related data.

Many tetracyclines, including DOX, have anti-inflammatory properties (41) mediated by suppression of tumor necrosis factor-α (TNF-α) and matrix metalloproteases (MMP). *In vitro*, DOX inhibits secretion of MMPs induced by TB infection at 5 mg/liter and higher (39). Thus, fine-tuning the DOX dose in both mouse and rabbit diets to reach but not exceed effective concentrations is important to minimize anti-inflammatory side effects and potential gastrointestinal disturbances.

MATERIALS AND METHODS

***In vitro* repression of Tet promoters.** The construction of the ATc/DOX-controlled *M. tuberculosis* mutants (Fum-DUC, Rho-DUC, and TrxB2-DUC) has been described (22–24). Mutant strains were grown to mid-log phase and diluted to an optical density (OD) of 0.03 in 7H9 medium. Bacteria were then exposed to 1.5-fold serial dilution of ATc or DOX. Optical density was recorded after 14 days and normalized to the corresponding strains without drug treatment.

Animal infection and DOX administration. All animal studies were performed in biosafety level 2 (BSL2) and BSL3 facilities and approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School, Rutgers University, Newark, NJ. All samples collected from *M. tuberculosis*-infected animals were handled and processed in the BSL3 facility in compliance with protocols approved by the Institutional Biosafety Committee of the New Jersey Medical School, Rutgers University, Newark, NJ.

For pharmacokinetic studies in uninfected mice, 4- to 6-week-old CD-1 female mice received a modified rodent diet containing 2,000 ppm DOX for the duration indicated in each experiment. Several diet lots from two different manufacturers were tested in pilot experiments. Laboratory rodent diet 5001 with DOX 2,000 ppm (DOX chow) from Purina (catalog number c113-200i) provided the highest and most consistent plasma levels and was selected for all experiments reported here. Blood was collected at

FOI 26-3132 - Document 5

various time points for up to 3 days while mice were receiving DOX chow, kept on ice, centrifuged at approximately 2,350 × *g* (5,000 rpm in a table-top centrifuge) for 5 min, and stored at −80°C until analyzed for DOX content.

For plasma and tissue pharmacokinetic studies in infected mice, 8- to 10-week-old female BALB/c mice were infected with a low dose of 100 to 200 CFU *M. tuberculosis* H37Rv (ATCC 27294) using a full-body inhalation exposure system (Glas-Col) as previously described (42). Mice received DOX chow for 7 days 4 weeks postinfection. For the determination of DOX content in major organs relative to plasma, one group of 3 mice was euthanized on day 7 and organs were collected, weighed, and stored at −80°C until analyzed for DOX content. Blood was collected at the time of euthanasia and processed as described above. To study the time to clearance of DOX from plasma and tissues after DOX chow (received for 7 days) was replaced with a DOX-free diet, groups of 3 mice were euthanized just prior to and then 1, 2, 4, and 7 h and 1, 2, 3, 4, 7, 10, and 14 days after replacement of DOX chow with standard rodent diet. Plasma and lungs were collected, processed, and stored as described above.

For pharmacokinetic studies in rabbits, female New Zealand white (NZW) rabbits (Millbrook Farm, Concord, MA), weighing 2.2 to 2.6 kg, were maintained under specific pathogen-free conditions and fed water and chow *ad libitum*. For dose-finding pharmacokinetic studies in uninfected animals, rabbits received DOX at the following doses/routes: 5 mg/kg intravenously formulated in saline, 20 mg/kg by oral gavage and formulated in deionized water, 24 mg/kg subcutaneously formulated in deionized water, or 15 and 30 mg/kg administered as DOX pills (either half a pill or one full pill of 100 mg doxycycline hyclate; Henry Schein Animal Health, Covetrus, OH) by oral gavage. Rabbit weights were approximately 3.0 to 3.5 kg at the time of dosing.

For plasma and tissue pharmacokinetics in TB-infected NZW rabbits, animals were infected with *M. tuberculosis* HN878 using a nose-only aerosol exposure system as described (43). At 12 to 20 weeks postinfection, once mature cellular and necrotic lesions had developed, rabbits received LabDiet 5001 supplemented with DOX at 200 ppm, 400 ppm, or 2,000 ppm (Research Diets, Inc., New Brunswick, NJ) for the duration indicated in each experiment.

For pharmacokinetic studies of DOX in a modified diet containing low or no calcium, uninfected rabbits received a gradually phased diet as follows. To test DOX PK in a calcium-free diet, rabbits received 25/75% calcium-free chow mixed with standard high-fiber rabbit chow (Laboratory Rabbit Diet HF 5326; LabDiet)—containing 10,000 ppm or 1% calcium—for 2 days, followed by 50/50% calcium-free chow mixed with standard chow for 2 days, then 75/25% calcium-free chow mixed with standard chow for 2 days, and finally, 100% calcium-free chow for 2 days, at which point they received a single oral dose of 25 mg/kg DOX formulated in deionized water (data shown in Fig. 3B). To test DOX PK in a low-calcium diet (20% of standard calcium content or 2,000 ppm), rabbits received 20/80% calcium-free chow mixed with standard high-fiber rabbit chow for 2 days, followed by 50% calcium-free chow mixed with 50% standard chow for 2 days, and finally, 80/20% calcium-free chow mixed with standard chow for 2 days, at which point DOX was added to the chow at 2,000 ppm final content (data shown in Fig. 3C). Customized calcium-free chow was manufactured with purified ingredients aiming to resemble the dietary parameters of standard high-fiber rabbit chow (18.2% protein, 56.2% carbohydrate, 3.6% fat; per kg of chow: 175 g casein, 390 g corn starch, 25 g maltodextrin, 125 g sucrose, 150 g cellulose, 25 g inulin, 35 g soybean oil, 38.3 g calcium-free mineral mix, 10 g vitamin mix, and 2 g choline bitartrate). Modified rabbit chow was obtained from Research Diets, New Brunswick, NJ.

***In vitro* pharmacokinetic assays and analytical methods.** Caseum binding and macrophage uptake of DOX were measured as described previously (31, 44).

High-pressure liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods for plasma and whole tissue analysis DOX-HCl and Verapamil were purchased from Sigma-Aldrich. DOX-d3 internal standard was purchased from Toronto Research Chemicals. Drug-free K_2EDTA plasma and lungs from CD1 mice and NZW rabbits were obtained from BioIVT for use as blank matrices to build standard curves. Neat 1 mg/ml dimethyl sulfoxide (DMSO) stocks DOX were serial diluted in 50/50 acetonitrile (ACN)/Milli-Q water to create neat standards. Control tissue homogenate and study sample homogenate were created by adding 9 parts phosphate-buffered saline (PBS) buffer to 1 part tissue (10× dilution) and shaking the samples using a Fisher bead mill for 1 min at 6,000 × *g* with zirconia beads. Standard, quality control, and study samples for DOX were extracted by adding 10 μl of tissue homogenate or plasma, 10 μl of DOX-d3 internal standard, and 100 μl of extract solvent containing 33% trichloroacetic acid. Extracts were vortexed for 5 min and centrifuged at 2,350 × *g* for 5 min. Then, 100 μl of supernatant was transferred to a 96-well plate for LC-MS/MS analysis. LC-MS/MS analysis was performed on a Sciex Applied Biosystems Qtrap 6500+ triple-quadrupole mass spectrometer coupled to a Shimadzu Nextera X2 ultrahigh-performance liquid chromatography (UHPLC) system to quantify each drug in plasma. Chromatography was performed on an Agilent Zorbax SB-C8 column (2.1 × 30 mm; particle size, 3.5 μm) using a reverse-phase gradient elution with aqueous mobile phase. Milli-Q deionized water with 0.1% formic acid (FA) and 0.1% heptafluorobutyric acid (HFBA) was used for the aqueous mobile phase, and 0.1% FA and 0.1% HFBA in ACN was used for the organic mobile phase. Multiple-reaction monitoring (MRM) of precursor/fragment transitions in electrospray positive-ionization mode was used to quantify the analytes. MRM transitions of 445.20/428.20 and 449.00/432.00 were used for DOX and DOX-d3, respectively. Sample analysis was accepted if the concentrations of the quality control samples were within 20% of the nominal concentration. Data processing was performed using Analyst software version 1.6.2 (Applied Biosystems Sciex).

Laser-capture microdissection coupled to LC-MS/MS. Laser-capture microdissection (LCM) sample quantification was carried out according to a previously published protocol (33). Briefly, 1 mg/ml neat DMSO stocks of DOX were serial diluted in 50/50 ACN/Milli-Q water to create neat standards. Control

FOI 26-3132 - Document 5

tissue homogenate was created by adding 25.6 parts PBS buffer to 1 part tissue (26.7× dilution) and shaking the samples using a Fisher bead mill for 1 min at 6,000 rpm with zirconia beads. Standard, quality control, and control samples were extracted by adding 2 μl of blank homogenate, 10 μl of neat standard, 5 μl of DOX-d3 internal standard, and 50 μl of extract solvent containing 33% trichloroacetic acid. LCM study samples were extracted that were identical to standards using 2 μl of PBS in place of tissue homogenate. Extracts were bath sonicated for 10 min and centrifuged at 2,350 × *g* for 5 min. Then, 50 μl of supernatant was transferred to a 96-well plate for HPLC-MS/MS analysis. HPLC-MS/MS analysis was performed as described above using the whole-tissue analysis methods.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.03 MB.

ACKNOWLEDGMENTS

This work was carried out with funding from grants OPP1182859 (D.S. and V.D.) and OPP1180610 (V.D.) from the Bill and Melinda Gates Foundation and NIH shared instrumentation grant S10OD023524 to V.D.

We thank the technical teams from the International Center of Public Health, Rutgers University, and the Center for Discovery and Innovation of Hackensack Meridian Health for their excellent support with all animal-related work.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

The AAPS Journal, Vol. 10, No. 1, March 2008 (© 2007)
DOI: 10.1208/s12248-007-9000-9

Mini-Review

The Pharmacokinetics and Interactions of Ivermectin in Humans—A Mini-review

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Received 14 September 2007; accepted 10 December 2007; published online 25 January 2008

Abstract. Ivermectin is an antiparasitic drug with a broad spectrum of activity, high efficacy as well as a wide margin of safety. Since 1987, this compound has a widespread use in veterinary medicine and it use has been extended in humans. Here we present a brief review of the information availabile regarding the pharmacokinetics and interactions of ivermectin in humans. Awareness of these characteristics could improve the clinical efficacy of Ivermectin. All Authors declare that they do not have any Conflict of interest and that the work is original. All Authors agree that the contents of the manuscript are confidential and will not be copyrighted, submitted, or published elsewhere (including the Internet), in any language, while acceptance by the Journal is under consideration.

KEY WORDS: humans; interactions; ivermectin; pharmacokinetics.

INTRODUCTION

Ivermectin is a semisynthetic derivative of avermectin B_1 and consists of an 80:20 mixture of the equipotent homologous 22,23 dehydro B_{1a} and B_{1b}. This antiparasitic agent, developed by Merck & Co., is frequently used in veterinary medicine, due to its broad spectrum of activity, high efficacy and wide margin of safety (1,2).

The first formulation destined to humans was launched in 1987, when Merck Laboratories had enough data to register ivermectin for use against onchocerciasis. The company announced that the drug would be provided at no cost to treat onchocerciasis, anywhere in the world, for as long as it was needed (3).

To control onchocerciasis, the *Onchocerciasis Control Programme in West Africa* was launched in 1974. The main goal of this program is to interrupt the parasite transmission cycle. Since 1987, the use of ivermectin in combination with aerial larviciding has had a remarkable impact on the transmission of the disease and greatly reduced the effect on humans. This led to the development, in 1992, of the *Onchocerciasis Elimination Programme in the Americas*, launched in 6 countries and, in 1995, of the *African Programme for Onchocerciasis Control*, both based mainly on distribution and treatment with ivermectin (4). In 1998, the *Global Programme to Eliminate Lymphatic Filariasis*, based on the regular mass administration of albendazole with either ivermectin or diethylcarbamazine, was initiated, confirming the safety and efficacy of the drug combinations (5).

Presently, ivermectin is approved for use in humans in several countries (Australia, France, Japan, The Netherlands, USA, etc.), to treat onchocerciasis, lymphatic filariasis, strongiloidiasis and/or scabies.

Ivermectin is exceptionally potent, with effective dosages levels that are unusually low. In the treatment of onchocerciasis, the optimal dose of ivermectin is 150 μg/kg, but the frequency of administration is still controversial, ranging from 150 μg/kg once to three times yearly. The optimal duration of treatment has not been established (6). It is effective in most patients with scabies after a single oral dose of 200 μg/kg, but often the regimen involves two or three repeated doses, separated by interval of 1 or 2 weeks (7).

Due to the extended use of this compound in humans, the knowledge of ivermectin pharmacokinetic behavior becomes essential. Nevertheless, little is known about the kinetics and interactions of ivermectin in humans compared to animals (even if the majority of fundamental work is veterinary, there is little evidence that such knowledge has helped to inform clinicians).

Thus, in this paper, we review the literature concerning the absorption, distribution, metabolism and excretion of ivermectin in man, as well as the interactions of the compound.

PHARMACOKINETICS

The oral route is the only approved for ivermectin administration in humans. The one-compartmental model (8, 9), as well as the two-compartmental model (10) have been used to describe ivermectin kinetic behavior after its oral administration. In the rest of the studies cited here,

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FOI 26-3132 - Document 5

pharmacokinetic parameters were determined using a model-independent method.

Absorption

In healthy subjects that received 12 mg of ivermectin as oral solution, tablets or capsules, it was shown that the solution had approximately twice the systemic availability as either of the solid forms (tablets and capsules showed similar systemic availability; Table 1). The oral solution was given as an ethanolic solution. This may affect bioavailability and could explain why the solution resulted in a twice as high availability as tablets and capsules. Nevertheless, the rate of absorption was similar in the three cases (11).

In healthy and onchocerciasis volunteers treated with 150 μg/kg, no significant differences were found in the pharmacokinetic parameters calculated. After an initial decrease, both groups showed a tendency for a second rise in plasma levels (mostly occurring between 6 and 12 h after the dose) suggesting an enterohepatic recycling of the drug (12).

It was also investigated whether diminished ivermectin absorption could explain the fact that some severely infected onchocerciasis patients experience relatively few adverse effects following ivermectin treatment, although the occurrence and extent of adverse reactions have been related to infection intensity (17). According to this study, there was no observable relation between the plasma concentration after a single oral dose (150 μg/kg) and the occurrence of adverse reactions. Neither parasite load nor ivermectin concentrations had an influence on the occurrence of adverse reactions in the entire group of patients.

The ability to achieve adequate levels of ivermectin after oral administration in patients with disseminated strongyloidiasis may be impaired, highlighting the need for alternative routes of administration of ivermectin in these patients. Thus, ivermectin levels, in a patient with disseminated strongyloidiasis, were below the average of those reported by other authors following oral administration (1.1 $ng \cdot ml^{-1}$ after ingesting a total dosage of 1,000 μg/kg over 3 consecutive days). This was followed by the administration of three subcutaneous doses (200 μg/kg), injected every 2 days, increasing ivermectin levels to 7.9 $ng \cdot ml^{-1}$ at 1 week after the last dose, with evidence of additional metabolite accumulation and also a sustained antiparasitic effect. Thus, in these patients, who are unable to absorb oral medication, parenteral ivermectin (not licensed for use in humans) is a better option (18). In another report, a man with disseminated strongyloidiasis, severe hypoalbuminemia and paralytic ileus, received ivermectin by the oral route. Three hours after the third daily dose, the serum ivermectin concentration was only 0.8 $ng \cdot ml^{-1}$, but it increased to 5.8 $ng \cdot ml^{-1}$ 16 hours after the first subcutaneous dose. Over the next 15 days, subcutaneous ivermectin (200 μg/kg, once a day) produced serum ivermectin levels between 11.4 and 17.2 $ng \cdot ml^{-1}$ with no significant accumulation (19).

Distribution

Due to the high lipid solubility of ivermectin, this compound is widely distributed within the body.

In healthy men, the volume of distribution in the central compartment, V_c, was 3.1 and 3.5 $l \cdot kg^{-1}$, after ingesting 6 and 12 mg of ivermectin, respectively (8). In onchocerciasis patients, with 6 mg (tablet), the volume of distribution of the area (V_λ) was 9.9 $l \cdot kg^{-1}$ and the mean residence time (MRT) was 3.7 days (16).

The tissue distribution of ivermectin was similar in healthy and onchocerciasis volunteers treated orally. So,

Table 1. Pharmacokinetic Parameters Obtained after Ivermectin Oral Administration

		Absorption			Elimination	
Reference	Dose	C_{max} ($ng \cdot ml^{-1}$)	t_{max} (h)	$t_{1/2(abs)}$ (h)	$t_{1/2}$ (h)	Cl ($1\ kg^{-1} \cdot day^{-1}$)
Healthy subjects						
9[a]	12 mg (tablet)	–	–	–	12	–
8[a]	6 mg (tablet)	23.1	4.3	0.5	12.6	4.28
8[a]	12 mg (tablet)	30.4	10.3	2.5	13.4	4.03
9[a]	6 mg (tablet)	20.2	4.7	1.4	11.1	7.57
9[a]	12 mg (tablet)	23.5	5.3	1.4	21.1	6.53
9[a]	18 mg (tablet)	31.2	5.1	1.7	16.7	10.6
11	12 mg (solution)	81	3.6	–	–	–
11	12 mg (tablet)	50	3.4	–	–	–
11	12 mg (capsule)	46	3.6	–	–	–
12	150 μg/kg	54.4	4.9	–	36.6	–
13	150 μg/kg	37.9	–	–	–	–
14	150 μg/kg	33.8	–	–		4.70 (♂) 8.40 (♀)
Onchocerciasis patients						
12	150 μg/kg	52.2	5.2	–	35.0	–
15	150 μg/kg	39	5.6	–	16	–
16	6 mg (tablet)	38.2	4.7	–	54.5	3.1
17	150 μg/kg				19.9	–

– Unknown data; C_{max} maximum plasma concentration; t_{max} time to reach C_{max}; $t_{1/2abs}$ absorption half-life; $t_{1/2}$ elimination half-life; *Cl* total body clearance

[a] One-compartment model

FOI 26-3132 - Document 5

infection with *Onchocerca volvulus* does not appear to affect the distribution of this drug. The compound was present in fat, skin, subcutaneous fascia and nodules and in worm fragments. Fat showed the highest and most persistent levels, whilst values for skin, nodular tissues, and worms were comparable, being the lowest concentrations found in the subcutaneous fascia (12). In patients with *Onchocerca volvulus* infection treated orally with ivermectin (150 μg/kg), Elkassaby (15) also detected the drug in the nodular tissue of the parasite and in worm fragments. After subcutaneous administration (500 μg/kg) to cattle with nodules of *Onchocerca ochengi* (a cattle parasite that can be used as a model of *O. volvulus*), high concentrations were detected in the capsule wall and inside the nodule (55 and 45 $ng \cdot g^{-1}$ on day 2; 60 and 30 $ng \cdot g^{-1}$ on day 7). So, the poor penetration of ivermectin into the nodules could not explain the lack of efficacy against adult filariae (20).

In the skin, the peak concentration of the drug in squames, sebum and sweat on the forehead and the antithenar was 8 h after a 12 mg oral dose and decline quickly beyond 24 h (21).

Ivermectin binds strongly to plasma proteins in healthy subjects (93.2%) (22). It was also high in onchocerciasis patients (93.1%), with a specific binding for serum albumin. There were two binding sites, with an association constant of 2×10^8 mol^{-1} at the primary site. Such avid binding assumes great importance as the drug is administered in world areas where malnutrition and hypoalbuminemia are common, so in these patients a decrease in plasma proteins and, consequently, a higher free fraction of ivermectin could be expected. Nevertheless, acetylsalicylic acid does not interfere with ivermectin binding (16).

Ivermectin was not detected in the cerebrospinal fluid of a man with disseminated strongyloidiasis, severe hypoalbuminemia and paralytic ileus, after five subcutaneous doses of ivermectin, when the serum level was 12.1 $ng \cdot ml^{-1}$ (18).

Elimination

Studies regarding the *metabolism* of ivermectin in humans are scarce. This drug is extensively metabolized by human liver microsomes by cytochrome P450. The predominant isoform responsible for the biotransformation of this compound in the liver of humans is cytochrome P-4503A4, converting the drug to at least 10 metabolites, most of them hydroxylated and demethylated derivatives (23). In plasma, radioactive metabolites were reported after the oral administration of ivermectin to healthy volunteers (10).

Data concerning *excretion* can be observed on Table 1. No differences in the elimination half-life were detected among healthy and onchocerciasis subjects (12). It was also suggested that the kinetics of ivermectin (its elimination half-life is around a day) were somewhat disconnected from its pharmacodynamics (antiparasitic events persisting for several months after a single dose of the drug) (11).

A significant effect of gender was found in ivermectin pharmacokinetics in healthy volunteers orally treated with 150 μg/kg, with a lower total body Cl/F in males compared to females (see Table 1) (14).

In connection with the excretion pathways, ivermectin and its metabolites were excreted mainly in faeces and only 1% in urine. Positive identification was obtained for the presence of 3"-*O*-desmethyl-H_2B_{1a}, and 22,23-dihydroavermectin B_{1a} monosacharide in urine and faeces, respectively [10]. Other authors who tried to determine ivermectin in urine (8, 9, 16) did not find the parent drug nor its metabolites.

Finally, in the milk of healthy women administered 150 μg/kg, the maximum concentration (14.1 $ng \cdot ml^{-1}$) was reached in 6.5 h (13). On the basis of this information, a breast-fed child would receive an average dose of only 2.75 μg/kg via milk. Thus, these authors did not recommend excluding breastfeeding mothers from mass ivermectin therapies, as they only represent 5–10% of the population in the areas hyperendemic with onchocerciasis.

INTERACTIONS

Several authors evaluated the effect of ivermectin coadministration with different drugs employed to control helminthes. To interrupt the transmission of onchocerciasis in humans, the combination of ivermectin and doxycycline is highly effective as, in infested patients, the ingestion of the anthelmintic (200 μg/kg, single dose) and the antibacterial (100 mg/kg, daily for 6 weeks), kept the microfiladermia levels low more time than did ivermectin alone. Doxycycline enhanced ivermectin-induced suppression of microfiladermia, as it sterilize adult female worms for a few months by depletion of symbiotic endobacteria of filariae, *Wolbachia* spp. (24) (essential for their survival and reproduction). Other studies showed that, to control onchocerciasis, the combination of ivermectin with other drugs offers no advantage over ivermectin alone. Thus, in infected patients, the ingestion of ivermectin had no effect on the kinetics of an oral dose of albendazole and there was no additive effect of both drugs against the parasite compared to ivermectin given alone (25, 26, 27). Albendazole (400 mg) did not modify the kinetic behavior of a single oral ivermectin dose (12 mg) (27). Also in onchocerciasis patients, the antiparasitary efficacy was similar when administering a single ivermectin dose (150 μg/kg, on day 1) followed by amorcazine (3 mg/kg twice daily, on days 8, 9 and 10) than when administering ivermectin alone (28). Similarly, the combination of ivermectin (200 μg/kg) and levamisole (2.5 mg/kg) was neither macrofilaricidal nor more effective against the microfilariae and the adult worms than ivermectin alone, although levamisole increased ivermectin plasma bioavailability in these patients (29).

The efficacy of drugs available for the treatment of infection with *Trichuris trichiura* is low in humans. Nevertheless, single-dose treatment with albendazole (400 mg)-ivermectin (200 μg/kg) appears to be highly effective against trichuriasis, being higher than when administering albendazole alone or with diethylcarbamazine (6 mg/kg) (30).

Thus, ivermectin interactions with another concurrently administered drugs can occur. This issue becames important, as combination chemotherapy is being used with increasing frequency as resistance to antiparasitic agents is becoming more widespread.

Regarding bleeding disorders, haematomatous swellings were reported in 2 out of 28 onchocerciasis patients treated with ivermectin (150 μg/kg), and prothrombin times were significantly above baseline by one week to one month after

drug ingestion, suggesting an antagonist effect against vitamin K (31). Nevertheless, in other 20 subjects, no changes were observed in prothrombin nor in thromboplastin times compared with baseline results, during 13 days after the ingestion of 220–420 μg/kg of ivermectin (32); bleeding disorders were not found in 15,000 patients treated with ivermectin (150 μg/kg) (33). Moreover, prolonged prothrombin ratios were observed in 148 subjects given ivermectin orally. Although no patients suffered bleeding complications, factor II and VII levels were reduced in most of them, suggesting interference with vitamin K metabolism. Ivermectin has a minimal effect on coagulation and concern about mass treatment for this reason appears to be unjustified (34). Finally, a man that had been on long-term oral anticoagulant therapy with acenocoumarol showed a persistent, excessive hypocoagulability while using insecticides (ivermectin and metidation) without protection to treat trees. So, it is to be taken into account that these types of interactions can exist and could cause hemorrhagic complications (35).

Information about the influence of foods in the pharmacokinetics of ivermectin is scarce. The knowledge of the influence of alcohol in ivermectin kinetic behaviour is scarce; co-ingestion of alcoholic drinks however is not recommended, because of ivermectin association with GABA receptors and the effect of alcohol in the central nervous system. In healthy volunteers administered ivermectin orally (150 μg/kg), plasma levels were significantly higher when coadministered with 750 ml of beer than with 750 ml of water; the plasma concentrations were significantly higher in patients who drank beer (66.3, 109, and 97.2 ng/ml at 1, 3 and 4 h, respectively) *vs*. those who drank water (44.0, 67.5, and 58.7 ng/ml, respectively, $P<0.01$ at each time point) (36).

Finally, ivermectin (150 μg/kg) was administered to 16 individuals with water or orange juice (750 ml). Orange juice decreased AUC (15.7 $ng \cdot d \cdot ml^{-1}$) and C_{max} (20.7 $ng \cdot ml^{-1}$) (water: 33.8 $ng \cdot ml^{-1}$; 24.3 $ng \cdot d \cdot ml^{-1}$), possibly because fruit juices and constituents are potent inhibitors of certain drug transporters (14).

CONCLUSION

Although the efficacy of ivermectin has been established in humans against several parasite diseases, the pharmacokinetic properties of this compound are less well known in humans compared to animals. Potential drug-drug interactions and drug-food interactions exist for ivermectin, which should be considered during therapeutic use of this drug.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Hospital Preparedness for COVID-19: A Practical Guide from a Critical Care Perspective

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Abstract

In response to the estimated potential impact of coronavirus disease (COVID-19) on New York City hospitals, our institution prepared for an influx of critically ill patients. Multiple areas of surge planning progressed, simultaneously focused on infection control, clinical operational challenges, ICU surge capacity, staffing, ethics, and maintenance of staff wellness. The protocols developed focused on clinical decisions regarding intubation, the use of high-flow oxygen, engagement with infectious disease consultants, and cardiac arrest. Mechanisms to increase bed capacity and increase efficiency in ICUs by outsourcing procedures were implemented. Novel uses of technology to minimize staff exposure to COVID-19 as well as to facilitate family engagement and end-of-life discussions were encouraged. Education and communication remained key in our attempts to standardize care, stay apprised on emerging data, and review seminal literature on respiratory failure. Challenges were encountered and overcome through interdisciplinary collaboration and iterative surge planning as ICU admissions rose. Support was provided for both clinical and nonclinical staff affected by the profound impact COVID-19 had on our city. We describe in granular detail the procedures and processes that were developed during a 1-month period while surge planning was ongoing and the need for ICU capacity rose exponentially. The approaches described here provide a potential roadmap for centers that must rapidly adapt to the tremendous challenge posed by this and potential future pandemics.

Keywords: pandemics; SARS virus; ICUs

As the novel coronavirus disease (COVID-19) pandemic emerged as a cause of profound respiratory failure in Wuhan, China (1, 2), healthcare facilities in the United States took notice. When the United States first reported cases in Washington state (3, 4) and epidemiologists estimated the potential impact on New York City hospitals, our institution (New York Presbyterian Hospital), like many other centers, prepared for an influx of critically ill patients. Excellent reviews of approaches to ICU preparedness in the setting of pandemics have been published, generally focusing on broad concepts of infection control, ways to increase staffing capacity, and community engagement (5). Similarly, others have highlighted the difficult issues involved in allocating scarce resources (6), particularly mechanical ventilators, in these challenging settings (7) (Table 1).

Given rapidly evolving data on the infectiousness of COVID-19 (8, 9), including virus transmission from asymptomatic or paucisymptomatic individuals (10, 11), the initial highest priorities included obtaining an adequate supply of personal protective equipment (PPE) (12) for the staff and evaluating and expanding ICU and ventilator capacity (13), among numerous other measures. As of this writing, there has been a rapid increase in the number of new cases, deaths, and healthcare use in New York State (Figure 1).

As the first patients arrived soon after planning began at our institution, surge plans for this pandemic were highly dynamic and iterative, requiring frequent updates and clear communication of changes. Here, we describe the procedures that were implemented over a 4-week time frame as patient admissions for COVID-19 increased exponentially. These approaches provide a potential roadmap for centers that must rapidly adapt to the tremendous challenges posed by a pandemic. We hope that even for medical centers with a much

(Received in original form April 10, 2020; accepted in final form April 16, 2020)

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Author Contributions: K.M.G., M.G.K., N.S.I., and L.L. made substantial contributions to the described protocols and drafted and revised the manuscript for important intellectual content.

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Am J Respir Crit Care Med Vol 201, Iss 11, pp 1337–1344, Jun 1, 2020
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Originally Published in Press as DOI: 10.1164/rccm.202004-1037CP on April 16, 2020
Internet address: www.atsjournals.org

Table 1. Solutions to Encountered Challenges

Challenge	Local Solution
Infection control	Define aerosolizing procedures; use guidelines to define appropriate PPE
Clinical challenges	
Decision to intubate	Evolving; determined by clinical judgment with no predefined criteria
Airway management	Intubation by RSI by most experienced operator; periintubation team to assist in gathering PPE and medications and to assist in transport
Engagement of infectious disease team	Universally consulted to assist with therapies and entry into clinical trials
Extracorporeal organ support	Patients evaluated by a multidisciplinary team on a case-by-case basis
Cardiac arrests	Policy limits the number of responders and promotes enhanced PPE
Standardization	Frequent conferences, daily e-mail updates, and shared files
Approaches to efficient use of time in the ICU	Create procedure team, proning team, and tracheostomy team; display results and patient data outside the room
Minimizing exposure	Bundle care, trial intravenous pumps, and ventilator monitors outside of the patient room
Education	Frequent, multidisciplinary conferences, and journal clubs
Communication with families	Daily calls to a patient surrogate; involvement of palliative care
ICU surge capacity	Increase COVID-19 capacity in multiple units simultaneously to expand expertise
Expansion to operating rooms	Multidisciplinary approach to understand differences between critical care ventilators and anesthesia machines
Efficient bed management	Appoint a clinical bed manager
Staffing	
Physician staffing	Create a model with ideal ratios of critical care physicians to ICU patients; use critical care experts from all backgrounds
Research faculty	Redeployed to clinical service if not doing COVID-19–specific research
Nursing and respiratory therapy	Rapid training in ICU skills; expedited recredentialing, education on newly introduced ventilators
Nonclinical staff	Acknowledge support from nonclinical staff
Ethical dilemmas	Use institutional ethics committees, with guidance from local laws, to approach each ethical decision
Staff wellness	Partner with mental health professionals for staff

Definition of abbreviations: COVID-19 = coronavirus disease; PPE = personal protective equipment; RSI = rapid sequence induction.

smaller footprint and lower surge capacity, some of the granular and practical advice provided here will aid others in rapidly adapting to this unprecedented challenge.

Background

Our campus of the greater New York Presbyterian system is jointly operated by physicians from Weill Cornell Medicine (medical college) and nurses, respiratory therapists, advanced practitioners, and nonclinical staff from New York Presbyterian Hospital. This campus is one of two quaternary centers within the hospital enterprise and receives transfers from smaller surrounding hospitals. We have six adult ICUs divided into subspecialties (medical, surgical, burn, cardiac, cardiothoracic, and neurosurgical), as well pediatric and neonatal ICUs and several step-down units.

Given previous recommendations (5, 6, 14), multiple categories were rapidly considered in preparation for a pandemic of massive scale, including infection control; clinical operational challenges; ICU bed surge capacity; adequate staffing of physicians, nurses, and respiratory therapists; complex ethical dilemmas; and staff wellness. Procedures to address these issues were created and implemented in collaboration with nursing, respiratory therapy, and hospital leadership.

Infection Control

Initial planning began in collaboration with hospital epidemiologists and infection control staff, and relied heavily on available information about the infectivity of COVID-19 (9, 15). These contributors, with guidance from the CDC (16), helped determine the appropriate PPE, define an "aerosol-generating procedure," and attempted to clarify and communicate the rapidly changing guidelines offered by the federal, state, and city governments. Importantly, hospital epidemiology personnel rapidly educated staff in COVID-19 units on the optimal format for donning and doffing PPE, and online videos and education materials were distributed hospitalwide.

Clinical Challenges

The Decision to Intubate

Given the initial impressions about the format for transmission of this novel coronavirus, the initial clinical approach was to avoid high-flow nasal cannula and noninvasive ventilation (9). When a patient who was under investigation or tested positive for COVID-19 experienced an oxygen saturation of ⩽88% on 6 L of oxygen via nasal cannula, intubation was recommended. Within 2 weeks, these

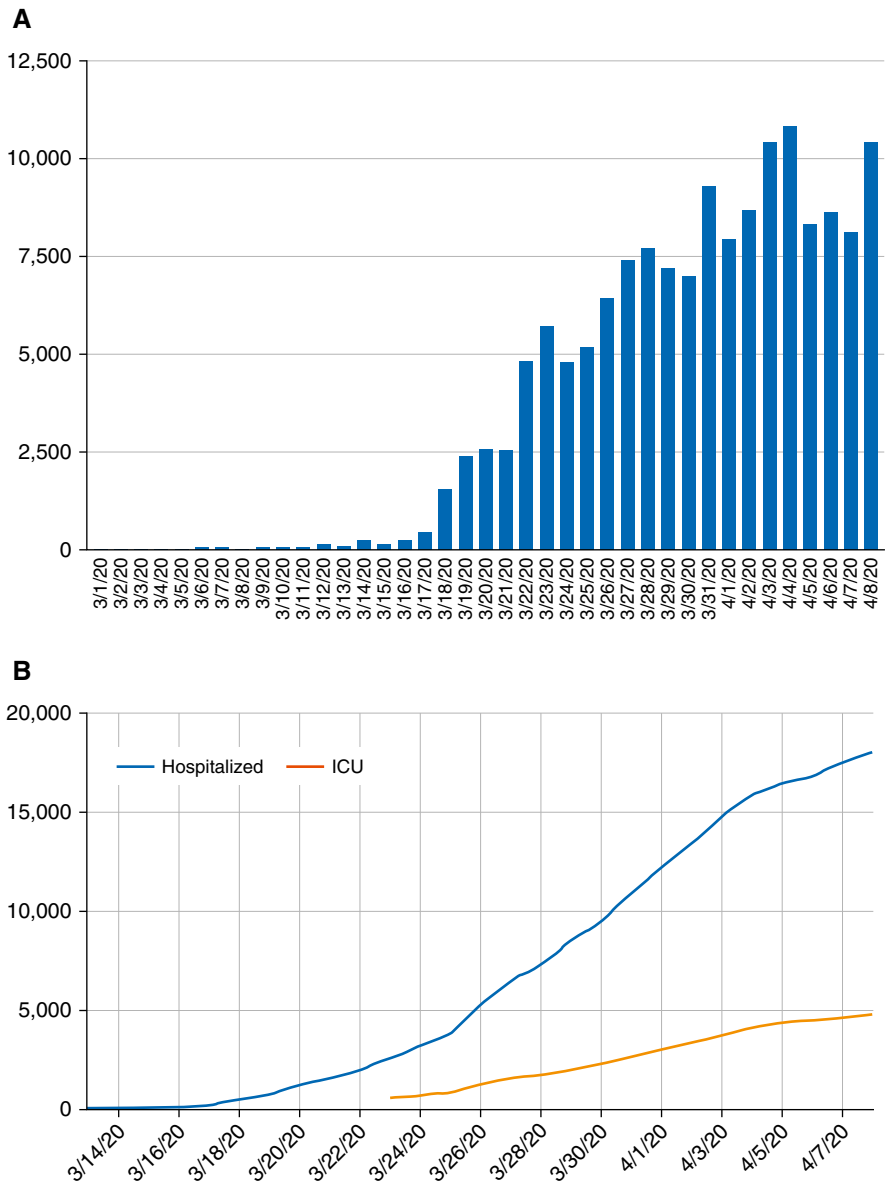


Figure 1. (*A*) New coronavirus disease (COVID-19) cases. (*B*) Total COVID-19 hospitalizations (blue) and ICU hospitalizations (orange) in New York State from March 14, 2020, to April 8, 2020. Reprinted by permission from Reference 34.

criteria were adapted to an oxygen saturation of <88% on 6 L of oxygen via nasal cannula in addition to a nonrebreather mask at 10 L of oxygen per minute. As the number of critically patients increased and international guidelines were refined, high-flow nasal cannula was increasingly used, preferably in a negative-pressure room (17), to possibly delay or avoid the need for intubation. The decision to intubate relies on the clinical judgment of the critical care physician and is based on factors such as hypoxemia, tachypnea, increased work of breathing, and gas exchange (18). These practices continue to develop as recommendations evolve.

Airway Management and the Intubation and Periintubation Teams

To maximize patient outcomes and minimize the risk to healthcare practitioners, a comprehensive airway policy was adapted from rapidly evolving international recommendations (19, 20). These procedures ensured that the most experienced operator would attempt intubation using rapid sequence induction (RSI) and a video laryngoscope. To enhance efficiency and optimize patient care, when a critical care physician identified a patient who required intubation, that physician notified the dedicated COVID-19 intubation team. Preparations included obtaining induction, sedation, and vasopressor medications; ensuring the use of appropriate PPE for this high-risk procedure (impervious gown, gloves, head covers, goggles or welder's mask, and optional shoe covers); and moving the patient to a negative-pressure room (with one reserved on each hospital floor) for intubation. The initial team included an anesthesiologist, a respiratory therapist, and a critical care nurse. The anesthesiologist intubated the patient using RSI and a video laryngoscope, and the multidisciplinary critical care team moved the patient to an ICU bed.

As the pace of hospital admissions and intubations accelerated, this process evolved. Two anesthesia airway teams were made available 24 hours a day, 7 days a week, dedicated to COVID-19 intubations. This team brought with them the appropriate PPE and a video laryngoscope. Hospital pharmacologists created virtual RSI kits, which could be rapidly accessed on every floor. Finally, a periintubation team led by certified registered nurse anesthetists assumed the role of coordinating periintubation procedures, including gathering medications and staff, and transporting patients to the negative-pressure room before intubation and to the ICU after intubation.

Engagement with Infectious Disease Consultants

Given the infectious nature of the disaster and the rapidly evolving therapeutic landscape, infectious disease consultants immediately established a dedicated COVID-19 team and pager. This team consults on every critically ill patient with COVID-19 daily to provide advice about available therapies (antiviral and antimicrobial) (21) and potential immunosuppressive approaches and innovative therapies (22, 23). Moreover, they serve as a gateway to rapidly evolving therapeutic trials (24).

Extracorporeal Organ Support

Institutional policies regarding extracorporeal membrane oxygenation are

FOI 26-3132 - Document 5

evolving with the publication of efficacy and risk data (25). Although extracorporeal membrane oxygenation has potential benefit in a subset of patients with COVID-19, the decision to cannulate must include the ability to provide appropriate staffing and resources, in addition to the usual criteria. Potential patients are discussed on a case-by-case basis by a multidisciplinary team. Renal replacement has been offered to all patients with acute or chronic renal failure via traditional hemodialysis, continuous venovenous hemodialysis, or peritoneal dialysis, with an awareness of the resources involved in each scenario.

Cardiac Arrests

Given the highly infective nature of the novel coronavirus, approaches to resuscitation have evolved (26, 27). As such, institutional cardiac arrest policies were instituted that limited the number of responders to cardiac arrests outside the ICU. Mechanical compression devices were rapidly introduced to further reduce the number of medical and nursing staff responding to an arrest. Medical ICU nurses, who respond to every cardiac arrest in our institution, carried a COVID-19 backpack to every cardiac arrest, which included high-risk PPE (welder-style face shields, N95 masks, and impermeable gowns) given the aerosolizing nature of cardiopulmonary resuscitation. Resuscitations in the ICU continue to be conducted in a standard fashion, with attempts to minimize aerosolization by leaving the patient on the ventilator or, if absolutely necessary, using a bag valve mask with a high-efficiency particulate air filter attached to the expiratory port.

Standardization of Care across All Critical Care Delivery Areas

Given the rapid expansion of ICU space and the broad number of caregivers involved in the care of critically ill patients with COVID-19, frequent formal and informal communication among all the groups has been essential. This is particularly important because some clinicians do not routinely care for patients with acute respiratory distress syndrome. Daily evaluation by a member of the pulmonary and critical care division is provided to all of the locations outside the medical ICU now caring for these patients. The development and distribution of informal guidelines outlining the granular aspects of caring for critically ill patients with COVID-19 have proved to be invaluable tools for providing standardized care in all areas. Secure electronic file sharing and daily e-mail updates give providers easy access to the latest hospital statistics and guidelines.

Approaches to Efficient Use of Time in the ICU

Dedicated smart phones with chargers are placed in each patient room so that staff members inside the room can contact additional staff outside the room without doffing their PPE.

A dedicated procedure team comprised of surgeons and interventional radiologists was created to perform ICU procedures such as central line, arterial line, or nasogastric tube insertion around the clock. This freed the intensivists to facilitate critical patient care. A multidisciplinary tracheostomy team led by thoracic surgery, otorhinolaryngology, and critical care specialists provides a similar focused approach to another pivotal aspect of critical care. A dedicated proning team was formed by specialists in critical care medicine and physical therapy to minimize the unit-specific staff needed for pronation or supination of patients while providing a standardized approach to this therapeutic maneuver (28). In an innovative adaptation, current drips, ventilator settings, and arterial blood gas results were written on the glass doors of the room so that staff could be informed without having to enter the room (Figure 2). This technique was made possible by the restriction of visitors to the hospital.

Minimizing Exposure to Staff

Care has been bundled as much as possible. Laboratory sampling was timed with medication dosing. Larger bags of intravenous fluid or more concentrated solutions were used to limit the number of times a nurse had to enter the room. Imaging studies and ECGs were limited to those deemed absolutely necessary. In some (but not all) rooms, intravenous pumps were placed outside the room with the use of extra intravenous tubing (Figure 2). Although this approach allows medications to be managed outside the room, its limitations include the length of time required for a bolus to travel to the patient. A buddy system including one staff member by the medication pumps became necessary to allow delivery of boluses or adjustments of medication to be made from outside the room while staff inside the room turned or cleaned the patient. The monitor for the most frequently used ventilators was detached and similarly kept outside the room (Figure 2). The advantages and drawbacks of this system mirror those of the intravenous pumps.

Education

Led by pulmonary and critical care physicians, a rapid simulation curriculum focused on pronation and COVID-19 cardiac arrests was instituted and provided for both day- and night-shift nurses in nonmedical ICUs. Our provider teams have been monitoring the rapidly evolving diagnostic and therapeutic approaches in the global arena to improve and optimize

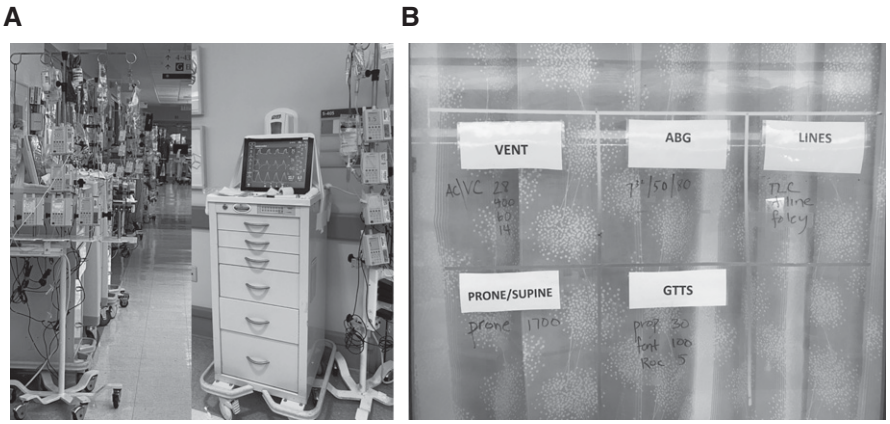


Figure 2. (*A*) Photos of ventilator screens and intravenous infusion pumps outside patient rooms. (*B*) Photo of patient data on the glass door of a patient's room. ABG = arterial blood gas; GTTS = guttae.

care on a local level. Remote-access conferences and journal clubs referencing seminal articles on acute respiratory distress syndrome continue to be conducted frequently. Topics have included fluid management, ventilator management, pronation, and sedation, among others. Recommendations from international colleagues are discussed in these multidisciplinary conferences.

Communicating with Families

The infectious nature of this pandemic has markedly impacted the ability of physicians and staff to communicate with patients' families, a key aspect of critical care. With limitations in visitation, family communication has become complex and fragmented. To address this aspect of patient care, a limited number of patient surrogates are designated to communicate with physicians. In addition, each family member is offered a second supportive call from a palliative care team member. The palliative care team discusses cases with each COVID-19 unit daily to assess for participating surrogates. In the setting of imminent death or immediately after death, a single family member in PPE is provided an opportunity to visit. If visitation is impossible, the staff can creatively use video conferencing to allow loved ones to visualize and speak to their dying family member.

ICU Surge Capacity

While creating ICU surge capacity, the first critically ill patients with COVID-19 were admitted to negative-pressure rooms divided among all of the ICUs. Initial surge plans were designed to accommodate an increased number of patients in the early stages (Figure 3), and later plans incorporated up to threefold higher numbers. Initially, for efficiency we designated half of the beds in multiple ICUs as COVID-19 units. This decision allowed units to acclimate to COVID-19 patients before the entire unit was filled, allowed staff members to perfect donning and doffing techniques, provided an opportunity to place pumps and ventilator monitors outside the room (Figure 2), and enabled multiple staff members to develop expertise in managing COVID-19. Having half of three separate ICUs simultaneously develop this expertise allowed a rapid doubling of ICU capacity.

Within 3 weeks of admitting our first critically ill patients with COVID-19, we had completely filled multiple ICUs. To respond to this challenge, less critical patients, when clinically feasible, were transferred to floor care, thereby freeing up the ICU beds. Subsequently, all critically ill patients without COVID-19 were grouped together in distinct or newly created ICUs. Elective surgeries were reduced to minimize surgical admissions and preserve PPE. As we entered the next phase of the surge plan, all cardiac patients without COVID-19 were grouped together in a new ICU. As units were opened and filled, additional units were rapidly identified. We began to care for patients with COVID-19 in operating rooms, with two ICU beds and two anesthesia machines per room. Similarly, step-down units and recovery rooms were rapidly converted to COVID-19 ICUs.

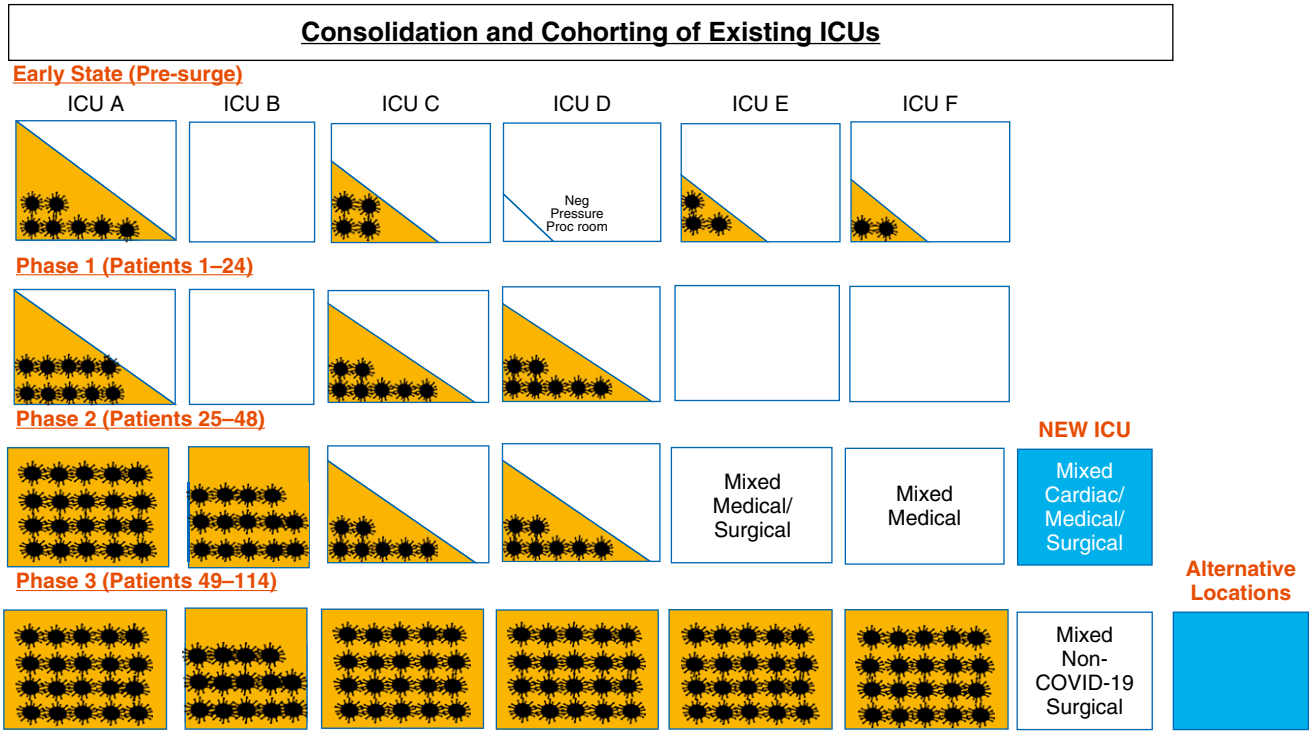


Figure 3. Each symbol denotes a patient with coronavirus disease (COVID-19). In the presurge period, patients were placed according to bed availability. Phase 1: unit readiness with partial COVID-19 census. Phase 2: start of ICU cohorting and creation of a new unit to displace patients without COVID-19 requiring specialty care. Phase 3: exhaustion of traditional ICU capacity. Patients who were not positive for COVID-19 were primarily postsurgical patients who were grouped together. Neg Pressure Proc room = negative pressure procedure room.

FOI 26-3132 - Document 5

Expanding to Operating Rooms

Expanding to operating rooms has created unique challenges, distinct from standard staffing and supply issues. Few caregivers outside of anesthesiologists and certified registered nurse anesthetists understand the intricacies of an anesthesia machine. The maximum flow rate of 15 L/min of fresh gas became important in patients with respiratory failure and a high $\dot{V}_E$ requirement. The proportion of fresh gas flow to recirculated air impacts the ultimate delivered $F_{I_{O_2}}$. Heat moisture exchangers may be added to the circuit but may not provide sufficient moisture to prevent desiccated secretions from occluding endotracheal tubes. Measuring airway pressures is challenging. In addition, providing nebulizers and checking inspiratory pauses on these machines requires breaking the circuit, which in general has been avoided in the interest of infection control.

Efficient Bed Management

Bed assignments and unit-to-unit transfers are typically managed by a centralized group of nurses in our patient placement operations center. This group also works in close collaboration with our transfer center, which organizes the incoming transfer of patients from other hospitals. During this time of rapid expansion, one of our critical care physicians partnered with these nurses around the clock, on a rotating basis, to triage all critically ill patients to streamline bed assignments and facilitate transfers into the newly created ICUs.

Staffing

Physician Staffing

ICU physician staffing has been rapidly expanded, and additional pulmonary and critical care physicians and critical care anesthesiologists have been redeployed to units with the goal of achieving a ratio of two senior physicians (with at least one being an intensivist) to 20 patients. The number of intensivists and critical care fellows in house each night rapidly increased fourfold with strong collaboration among the pulmonary and critical care, cardiology, neurointensive care, surgical critical care, and anesthesia teams. Twice-daily huddles occurred among all in-house leaders in critical care, including the airway team, to coordinate care and facilitate interdisciplinary teamwork. Additional hospitalist, trainee, and physician-extender teams were redeployed to provide patient care around the clock, as well as to provide medical expertise to nonmedical intensivists.

Research Faculty

All research activities at the medical college have been drastically curtailed to free up caregivers for clinical work. Ongoing research is limited to that which is focused on COVID-19. In addition, multiple clinical trials for possible therapeutic agents have been approved by the institutional review board and are enrolling patients.

Nursing and Respiratory Therapy Staffing

In phase 1 of the surge plan, medical and step-down nurses began training and shadowing in ICUs. Training fairs to teach ICU skills were held daily to help prepare nurses for caring for the critically ill. To facilitate staffing, nurses who previously worked at our institution were contacted and offered expedited recredentialing if they were willing and available. Respiratory therapists are an invaluable part of the care team for patients with respiratory failure. Given the pressures on the healthcare system, it has been challenging to rapidly and adequately increase the number of respiratory therapists. Critical care faculty and trainees have assisted in ventilator management. As the standard fleet of critical care ventilators became limited, transport ventilators were obtained and operationalized for ICU care. Because intensivists are less familiar with these devices, respiratory therapists created innovative approaches to rapidly educate clinicians in their use.

Nonclinical Staff

Members of the institutional facilities staff have played a key role in rapidly creating innovative barriers and negative-pressure rooms. The environmental services staff members became expert at donning and doffing PPE and clearing refuse at an accelerated rate in COVID-19 rooms owing to the increased PPE requirements. They devised impressive approaches to rapidly turn over rooms that are vitally required for aerosolizing procedures. It is evident that every staff member plays a key role in rapidly expanding ICU capacity and care in the setting of a highly infective agent in the setting of a pandemic. Within days of the first COVID-19 admission to our institution, our colleagues in laboratory medicine implemented in-house testing, allowing us to rapidly scale the number of patients we could test each day.

COVID-19 poses a potential infectious risk to all personnel in the hospital, and protection of staff has remained a top priority. Adequate PPE has been provided to all staff. In collaboration with the Division of Infection Control and Prevention, the hospital and medical college have ensured appropriate education on donning and doffing of PPE and precautions regarding aerosolizing procedures.

Ethical Dilemmas

The rapid and unprecedented growth in the number of critically ill patients (Figure 1) raised the specter of difficult ethical decisions. Several groups globally have provided general guidelines regarding resource allocation in the setting of a challenging pandemic (14, 29, 30). Given the increased negative outcomes in older individuals or those with multiple comorbid illnesses (31, 32), the need for discussions with palliative care and ethics consultants has become more acute. We used the New York State guidelines as a framework for these conversations (33). We have encouraged our colleagues in emergency medicine and hospital medicine to pursue early, honest conversations with patients and families regarding goals of care and clarifying advance directives. A program that provides access to palliative care and ethics consultants, 24 hours a day, has helped facilitate these conversations.

Staff Wellness

This challenging pandemic has created tremendous moral distress among healthcare personnel. We pride ourselves on the personalized care of our patients. One challenging aspect of care in this pandemic is the inability to provide care in the usual manner, which normally involves including patients' families as their support system throughout their illness. Staff members have facilitated emotional, life-altering conversations over video chats with family members and have watched families say

goodbye to each other without the benefit of privacy; the staff cannot leave the room while they hold the phone or tablet. Physicians and nurses work to balance being present and offering comfort while minimizing their personal exposure to COVID-19. In addition to this heartbreaking reality, we work while watching this pandemic take over our city. Staff fear for their own health and that of loved ones. Many providers have isolated themselves from their families, either by staying in hotels or by sending their families away. Some have created or updated wills and advance directives. For all of these reasons and because of sheer exhaustion, we have seen our staff become overwhelmed with emotions ranging from anxiety to helplessness. To deal with these challenges, it has been vital to engage mental healthcare providers with expertise in the management of trauma and acute stress to offer individual and group support to our staff. Referrals have been made as needed for psychological support, and staff members have been given 24-hour direct access to a mental health professional.

Conclusions

As New York City has become the current global epicenter of the COVID-19 pandemic, our institution has creatively expanded our caregiving capabilities in multiple ways. This outline provides an understanding of how we, in collaboration with and appreciation of our colleagues in the departments and divisions of pulmonary and critical care, anesthesiology, cardiology, neurocritical care, surgical critical care, hospital medicine, infectious diseases, palliative care, ethics, nursing, physical therapy, and respiratory therapy, are meeting this challenge. We provide potential guidelines for other centers to adapt as needed to help further streamline this expansion. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Dr. Kate Heilpern for her tremendous support and leadership during this challenging period. They also thank their partner physicians in pulmonary and critical care, cardiology, anesthesiology, surgery, neurology, infectious diseases, and hospital medicine, and the medicine trainees, physician assistants, and nurse practitioners who have worked tirelessly for their patients. They acknowledge the nurses, respiratory therapists, physical and occupational therapists, chaplains, aides, and other front-line staff for their dedication to patient care. The authors could not have accomplished this work without the aid of their colleagues in environmental services, facilities, patient transport, and procurement, among countless others.

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FOI 26-3132 - Document 5

Tetracyclines: a pleitropic family of compounds with promising therapeutic properties. Review of the literature

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[1]*Transitional Year Residency Program, Wheaton Franciscan Healthcare-St. Joseph, Milwaukee, Wisconsin;* [2]*Department of Medicine, University of California, San Diego, California; and* [3]*Escuela Superior de Medicina, Instituto Politecnico Nacional, Mexico, DF, Mexico*

Submitted 11 February 2010; accepted in final form 27 June 2010

Griffin MO, Fricovsky E, Ceballos G, Villarreal F. Tetracyclines: a pleitropic family of compounds with promising therapeutic properties. Review of the literature. *Am J Physiol Cell Physiol* 299: C539–C548, 2010. First published June 30, 2010; doi:10.1152/ajpcell.00047.2010.—There must be something unique about a class of drugs (discovered and developed in the mid-1940s) where there are more than 130 ongoing clinical trials currently listed. Tetracyclines were developed as a result of the screening of soil samples for antibiotic organisms. The first of these compounds chlortetracycline was introduced in 1948. Soon after their development tetracyclines were found to be highly effective against various pathogens including rickettsiae, Gram-positive, and Gram-negative bacteria, thus, becoming a class of broad-spectrum antibiotics. The mechanism of action of tetracyclines is thought to be related to the inhibition of protein synthesis by binding to the 30S bacterial ribosome. Tetracyclines are also an effective anti-malarial drug. Over time, many other "protective" actions have been described for tetracyclines. Minocycline, which can readily cross cell membranes, is known to be a potent anti-apoptotic agent. Its mechanism of action appears to relate to specific effects exerted on apoptosis signaling pathways. Another tetracycline, doxycycline is known to exert antiprotease activities. Doxycycline can inhibit matrix metalloproteinases, which contribute to tissue destruction activities in diseases such as gingivitis. A large body of literature has provided additional evidence for the "beneficial" actions of tetracyclines, including their ability to act as oxygen radical scavengers and anti-inflammatory agents. This increasing volume of published work and ongoing clinical trials supports the notion that a more systematic examination of their possible therapeutic uses is warranted. This review provides a summary of tetracycline's multiple mechanisms of action and while using the effects on the heart as an example, this review also notes their potential to benefit patients suffering from various pathologies such as cancer, Rosacea, and Parkinson's disease.

protease inhibitors; doxycycline; minocycline

Classical Uses of Tetracyclines

THE TETRACYCLINES are an aging family of broad-spectrum antibiotics. The parent compound chlortetracycline (tradename Aureomycin) was first isolated from *Streptomyces aureofaciens* in 1947 (21). Soon after, other natural tetracyclines were isolated, including tetracycline, for which the family of molecules is named. Since then, the modifications of naturally occurring tetracyclines and the synthesis of novel compounds within the tetracycline family have generated many compounds. Two of the more common semisynthetic tetracyclines used clinically as antibiotics are doxycycline (DOX) and minocycline (MIN), which are essentially well-tolerated and safe compounds. Because of its broad-spectrum antibiotic efficacy, DOX is indicated for the treatment of a variety of infections, including anthrax, chlamydial infections, community-acquired pneumonia, Lyme disease, cholera, syphilis, *Yersinia pestis* (plague), periodontal infections, and others. MIN also displays broad-spectrum efficacy and is most often used clinically in the treatment of severe acne, but it is also indicated for many of the same infections as DOX (50).

The tetracyclines exert their antibiotic effect primarily by binding to the bacterial ribosome and halting protein synthesis (39). Bacterial ribosomes have a high-affinity binding site located on the 30S subunit and multiple low-affinity sites on both the 30S and 50S subunits (89). Upon binding the ribosome, the tetracyclines allosterically inhibit binding of the amino acyl-tRNA at the acceptor site (A-site), and protein synthesis ceases (76). However, the use of tetracyclines has declined in recent decades due to the emergence of resistant strains of bacteria (Fig. 1). The primary mechanism of resistance is mediated by increased drug efflux out of the cell by a family of Tet proteins located on the cytoplasmic surface of the cell membrane (55, 97). Whereas an understanding of the mechanisms of bacterial resistance (42) to the tetracyclines is important clinically, it also has led to the development of the

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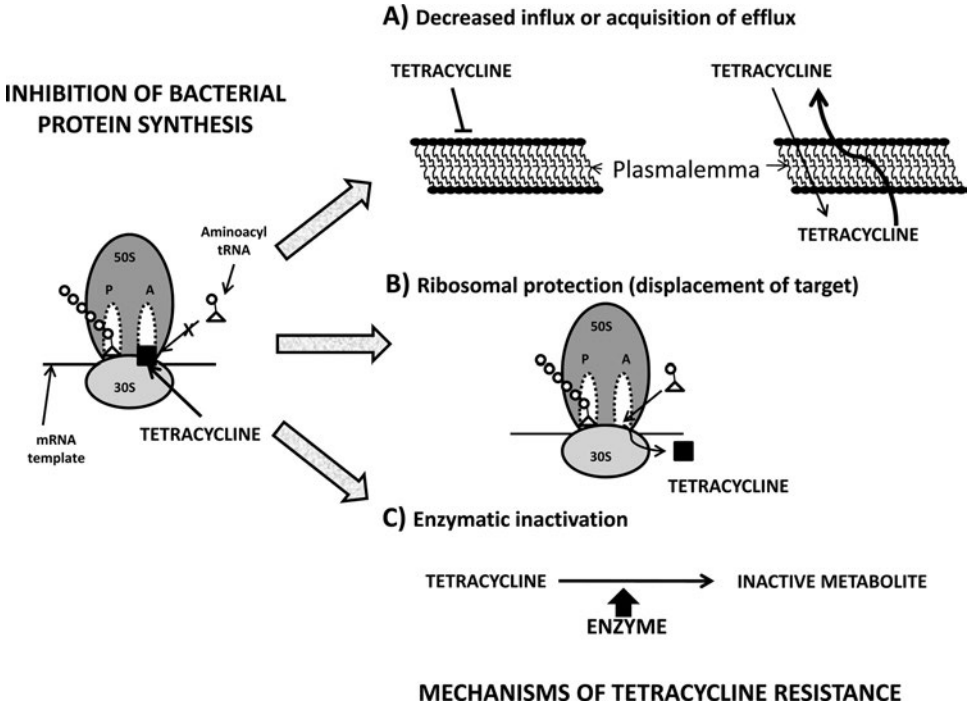


Fig. 1. Illustration of the means by which tetracyclines may lose their protein synthesis inhibitory capacity in pathogens such as bacteria. This may occur secondary to the enhanced extrusion of the drug, decreased entry, displacement from the target (ribosomes), or enzymatic inactivation.

Tet regulatory system, an important transcriptional regulation tool that is used extensively for targeted gene regulation in eukaryotes (35).

Tetracyclines are also effective but slow-acting anti-malarial drugs. DOX-treated parasites appear morphologically normal until late in the second cycle of treatment but fail to develop into merozoites (15). DOX specifically impairs the expression of apicoplast genes. Apicoplast (nonphotosynthetic major organelles found in cells of plants) are abnormal in the progeny of DOX-treated parasites. The loss of apicoplast function in the progeny of treated parasites leads to a slow but potent anti-malarial effect.

Chemical Properties

While the complex chemistry of the tetracyclines is beyond the scope of this review, a basic understanding of the important features of these compounds is necessary to appreciate their ability to interact with such a wide variety of biomolecules. Tetracycline, DOX, and MIN are all composed of a four-ring core to which are attached various side groups (Fig. 2). The dimethylamino group at the C4 carbon on the upper half of the molecule has been shown to be necessary for antimicrobial activity. 4-De-dimethylamino tetracyclines, also called chemically modified tetracyclines (CMTs), lack antimicrobial activ-

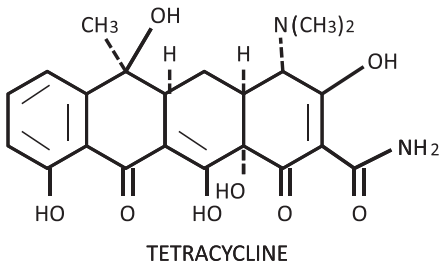
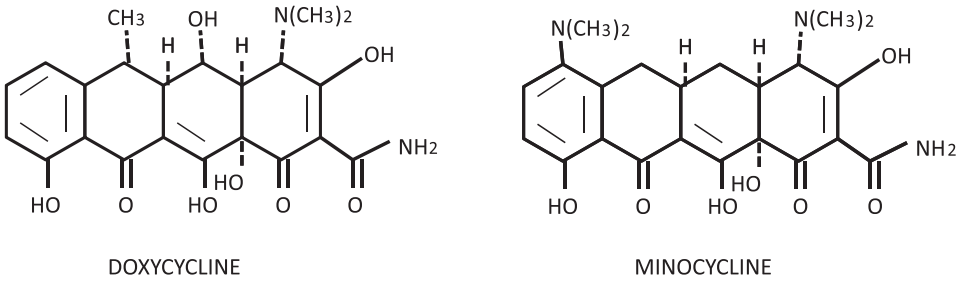


Fig. 2. Chemical structure of tetracyclines.



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ity in vivo presumably due to the inability of the molecule to adapt a zwitterionic form necessary for activity (56). However, CMTs do retain the ability to bind other nonmicrobial targets, such as matrix metalloproteinases (MMPs), facilitating their use in the treatment of other disease processes (32). The oxygen-rich lower half of the molecule is critical for binding to both prokaryotic and eukaryotic targets, and interference with this region reduces or eliminates the effectiveness of the drug (33). This region is relevant as site for metal ion chelation. Binding of tetracyclines to proteins, including TetR, may be greatly enhanced when the tetracycline is complexed with divalent metal ions such as Ca^{2+} or Mg^{2+} (86). The binding of tetracyclines to MMPs is thought to be mediated by the chelation of structural and catalytic Zn^{2+} ions within the enzyme (Fig. 3) (33, 70). In addition, binding to the bacterial ribosome involves binding to RNA-bound Mg^{2+} (29). The strength of tetracycline-metal interaction is dependent on both the tetracycline and the metal ion present. In general, the affinity of the tetracyclines for different divalent metals is in order of decreasing affinity: $Cu^{2+} > Co^{2+} = Fe^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$ (61). The relative affinities of different tetracyclines for a given metal also differ and are highly dependent on pH and the presence of other metal ions (4, 6, 53). The relative superiority of DOX as an MMP inhibitor is due to its increased affinity for Zn^{2+} compared with tetracycline or MIN (7). In general, there is a direct relationship between lipophilicity and activity against Gram-positive bacteria. The lipophilicity of tetracycline, DOX, and MIN, as determined by partitioning between octanol and aqueous buffer, has been determined to be 0.025, 0.600, and 1.1, respectively (11), and the minimum inhibitory concentration against *Staphylococcus aureus* is 0.21, 0.19, and 0.10 μg/ml, respectively (5). Lipophilicity also affects tissue distribution. MIN is able to cross the blood-brain barrier much more readily than DOX or tetracycline. MIN attains levels in the brain nearly threefold higher than DOX, and tetracycline is undetectable in the brain (3).

Matrix Metalloproteinase Inhibition by Tetracyclines

Probably the best characterized non-antimicrobial property of the tetracyclines is their ability to inhibit members of the MMP family of endopeptidases. The MMPs are a family of zinc-dependent proteases that are involved in many physiological and pathophysiological processes including embryogenesis, tissue remodeling, inflammation, and tumor invasion (58). MMPs can be subdivided based on crude substrate specificities into the collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs) (82). The collagenase group includes MMP-1, MMP-8, and MMP-13, and all cleave fibrillar collagens (types I and III). Collagen fragments subsequently denature into gelatins. The gelatinases, which include MMP-2 and MMP-9, proteolyze the gelatins. The gelatinases also degrade basement membrane collagen (type IV). The stromelysins includes MMP-3, MMP-7, MMP-10, and MMP-11, and they are capable of degrading proteoglycans, laminin, fibronectin, collagen IV, and others. The cell membrane-anchored MT-MMP include six different MMP, of which MT1-MMP is the best characterized (19).

Inhibition of MMPs is beneficial in many pathological conditions in which MMP-mediated proteolysis of the extracellular matrix (ECM) contributes to the pathogenesis, such as heart remodeling, tumor invasion, and inflammation (58, 64, 67). Numerous synthetic compounds with potent and specific in

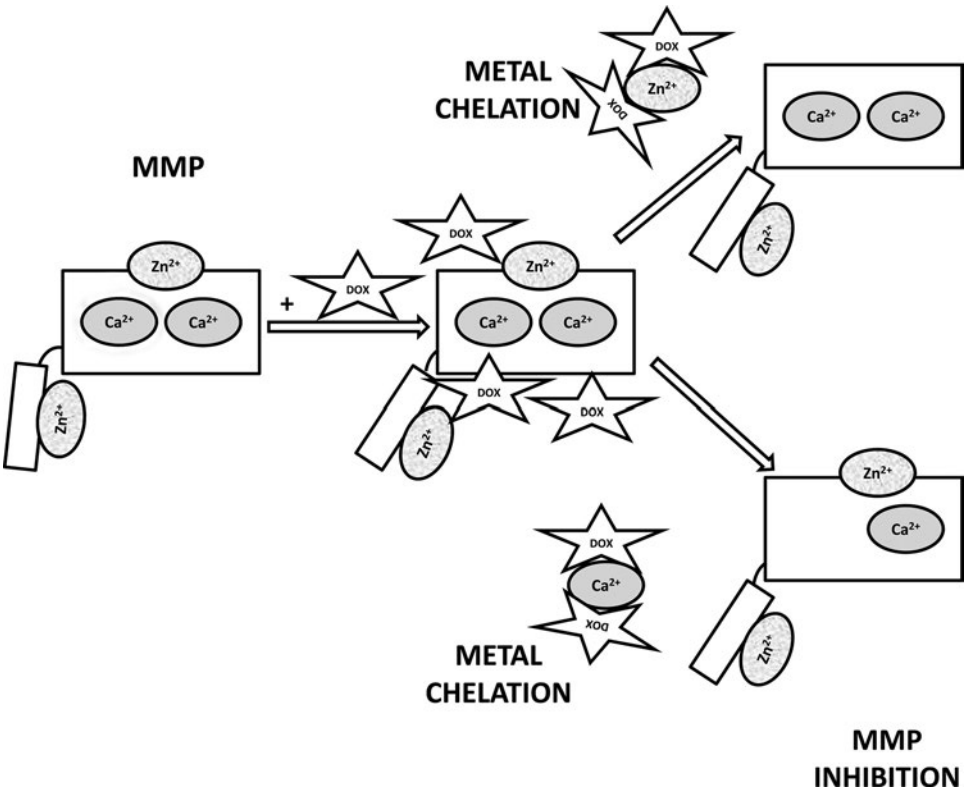


Fig. 3. Illustration of the means by which doxycycline through zinc and calcium chelation may act to inhibit matrix metalloproteinases (MMPs). Both ions are crucial to allow for enzyme activity.

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vitro MMP inhibitory activities were developed, and many have entered clinical trials (64). However, because of the lack of efficacy and serious musculoskeletal side effects, none progressed to phase III clinical trials. Currently, the only clinically available MMP inhibitor is DOX, which is marketed as Periostat, and it is indicated only for the treatment of periodontitis (64). Following the seminal experiment by Golub et al. (31) showing that MIN inhibited collagenase derived from rat gingiva, tetracyclines became increasingly studied for their ability to inhibit MMPs.

The mechanism by which tetracyclines inhibit MMPs has not been completely elucidated. It is believed that they exert their anti-proteolytic effects by both direct inhibition of MMPs and by inhibiting their expression. Direct inhibition of MMPs appears to be mediated by an interaction between the tetracycline molecule and metal ions within the MMP. MMP inhibition can be reversed by the addition of millimolar amounts of Ca^{2+} (30) or micromolar amounts of Zn^{2+} (100). Interestingly, it appears that the mechanism of inhibition is dependent on chelation of structural metals rather than chelation of the active site Zn^{2+} (78). In addition, it was recently shown that DOX binds to MMP-7 near the structural Zn^{2+} and Ca^{2+} ions and does not bind to the catalytic Zn^{2+} (28) (Fig. 3). The effectiveness of tetracycline inhibition against various MMPs depends on the tetracycline species, MMP species, and the pH. It has been shown that DOX is more potent than MIN or tetracycline against collagenases purified from rabbit corneas, with IC_{50} values of 15 μM, 190 μM, and 350 μM, respectively, and this trend may be explained by the relatively high affinity of DOX and low affinity of tetracycline for Zn^{2+} (7). The IC_{50} values for DOX against the collagenases MMP-8, MMP-13, and MMP-1 is 1–10 μM, 5–30 μM, and >200 μM, respectively (36, 34, 79); the reasons for the differences in inhibition of the various MMPs are not clear. The pH of the system also affects inhibition as evidenced by the ability of DOX to inhibit MMP-8 at pH > 7.1 and inability to inhibit at pH < 7.1 (77). In addition to inhibiting MMPs directly, tetracyclines also inhibit MMP synthesis. DOX inhibited cytokine-induced MMP-8 mRNA and protein accumulation in cultured rat synovial fibroblasts (41). In cultured human skin fibroblasts, tetracycline inhibited interleukin-1 (IL-1)-induced MMP-3 expression (48). Since MMP transcription is induced by a host of proinflammatory cytokines and other growth factors, including IL-1, IL-6, tumor necrosis factor-α (TNF-α), epidermal growth factor, and others (13), it is likely that these upstream signaling cascades leading to MMP expression are important targets of tetracyclines. An interesting consequence of MMP inhibition is the indirect inhibition of serine proteases. MMPs can inactivate serine protease inhibitors (SERPINs) (17, 57), and MMP inhibition with DOX or CMTs preserves SERPINs thereby blocking serine protease activity (14, 30, 37, 80, 81).

MMP activity is upregulated early following a myocardial infarction (MI), suggesting an important role in mediating the acute injury and healing processes of the heart, including inflammation, angiogenesis, scar formation, and remodeling. However, early MMP activity may be a double-edged sword, leading also to early damage of the cardiac ECM. MMP activity increases within minutes of ischemia, reflecting activation of preformed latent MMPs within the myocardium (16, 23, 68). The simultaneous appearance of type I collagen fragments in serum indicates that this early MMP activity contributes to significant collagen proteolysis (90). This early damage may set the stage for long-term adverse remodeling. In addition to their role in mediating post-MI healing and remodeling, there is emerging evidence that the MMPs may actually contribute to myocyte death and dysfunction following ischemia-reperfusion (I-R) injury. MMP-2 activity is increased in isolated, perfused rat hearts following I-R, and treatment with DOX attenuated the increase in MMP-2 activity upon reperfusion and improved the recovery of contractile function (10). MMP-2 activation following I-R resulted also in proteolysis of troponin I and myosin light chain, and DOX prevented their proteolysis (74, 94). It is possible that MMP-mediated disruption of the normal myocyte-matrix architecture leads to myocyte cell death, since disruption of normal cell-ECM interactions has been shown to contribute to the induction of myocyte apoptosis both in vitro (71) and in vivo (18). These studies demonstrate that inhibition of MMP activity holds promise for salvaging myocytes and preserving cardiac structure and function post-MI. However, for pharmacological MMP inhibition to be beneficial, the timing of MMP inhibition will likely be important. For example, early short-term MMP inhibition post-MI (<48 h) may block the early damage to the ECM while still allowing later events (>48 h), such as inflammation and wound healing, to proceed normally. Indeed, significant anti-remodeling (i.e., beneficial) effects can be observed in the infarcted heart when DOX is given for a limited period of time after injury (27).

Reactive Oxygen Species Scavenging by Tetracyclines

Another well-characterized nonantimicrobial property of the tetracyclines is their ability to scavenge reactive oxygen species (ROS). Excess ROS are produced under many pathological conditions, including myocardial I-R injury and inflammatory processes, and can lead to the oxidative destruction or dysfunction of many cellular constituents. ROS are highly reactive and include the free radicals superoxide ($O_2^{\cdot -}$) and hydroxyl radical ($OH^{\bullet}$), the non-free radicals hydrogen peroxide (H_2O_2), hypochloride (HOCl), and peroxynitrite ($ONOO^-$) (62). DOX, MIN, and tetracycline all have a multiple-substituted phenol ring, similar to vitamin E. The phenol ring is key to the ROS-scavenging abilities of these compounds. The reaction of the phenol ring with a free radical generates a phenolic radical that becomes relatively stable and unreactive due to resonance stabilization and steric hindrance by the phenol ring side groups (52). MIN directly scavenge ROS in several cell-free mixed-radical assays with a potency comparable to vitamin E, and its scavenging ability was not dependent on chelation of Fe^{2+} (i.e., inhibition of the Fenton reaction) (52). Depending on the assay used, MIN had an IC_{50} of 3–40 μM and is 9–250 times more potent as scavenger than that of DOX and 200–300 times more potent than of tetracycline. The superior scavenging ability of MIN is likely due to the presence of the diethyamino group on the phenolic carbon (Fig. 4), which is unique to MIN and provides improved steric hindrance (52). Other groups have shown DOX to be an effective ROS scavenger. DOX inhibits both HOCl and a mixture of neutrophil-derived ROS in vitro, with an IC_{50} of ~2 μM (43). In addition, DOX inhibits HOCl-mediated activation of MMP-1 from osteosarcoma cells, with an IC_{50} of ~50 μM (66).

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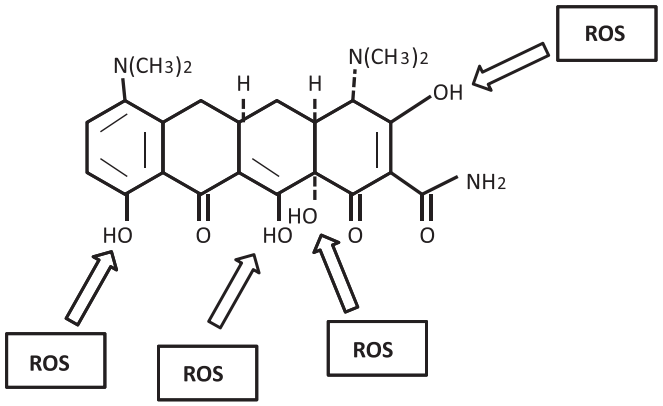


Fig. 4. Illustration highlights minocycline hydroxyl radicals that may react with reactive oxygen species (ROC) thus yielding antioxidant activity.

The generation of ROS upon reperfusion of ischemic myocardium is thought to be one of the primary mediators of reperfusion injury. It has also been shown that administration of exogenous SOD (9, 93, 99) and/or catalase reduces the extent of I-R injury in canine (1, 47) and porcine (60) models. The addition of $ONOO^-$ (which degrades to NO_2 and $OH^\bullet$) to isolated adult cardiac myocytes caused impaired calcium homeostasis and contractile dysfunction (54). DOX treatment normalized calcium transients and reduced the decline in contraction induced by $ONOO^-$.

Anti-Apoptotic Effects of Tetracyclines

A key event in the execution of the apoptotic cascade is the activation of caspases, a family of cysteine proteases. Caspases can be divided into upstream "initiator" caspases, which function to activate downstream caspases, and the downstream "executioner" caspases that are responsible for the proteolytic destruction of the cell (25). Caspases may be activated by either the extrinsic (death receptor) pathway or the intrinsic (mitochondrial) pathway. In the extrinsic pathway (88), death signal proteins such as TNF-α and Fas ligand bind to their cell surface receptors and, through signal transduction cascades, lead to the activation of an initiator caspase caspase-8. Caspase-8 in turn activates downstream caspases, such as caspase-3. In the intrinsic pathway (72), a variety of extracellular and intracellular stress stimuli converge on the mitochondria to destabilize it and cause the release of pro-apoptotic factors into the cytosol. A key factor mediating mitochondrial destabilization is opening of the mitochondrial permeability transition pore (mPTP), a large, high-conductance pore that spans both the inner and outer mitochondrial membranes (40). Once the mPTP opens, there is an influx of water, which leads to swelling of the mitochondrial matrix and eventually rupture of the outer mitochondrial membrane. Cytochrome c and other pro-apoptotic proteins, such as Smac/DIABLO [mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein (IAP) binding protein with low PI]and apoptosis-inducing factor (AIF) are released from the intermembrane space into the cytosol. Cytochrome c binds the cytosolic adapter protein Apaf-1, which allows the recruitment and activation of an initiator caspase caspase-9, which then activates downstream caspases (46). Smac/DIABLO binds and inhibits caspase inhibitor proteins thereby disinhibiting caspase activity (59). AIF translocates to the nucleus and induces chromatin condensation and DNA fragmentation in a caspase-independent manner (85). The mitochondrial apoptosis pathway is also partly regulated by the Bcl-2 family of proteins (12). Bcl-2 family members may be pro-apoptotic (Bax, Bad, Bid) or anti-apoptotic (Bcl-2, Bcl-xL).

There is an increasing body of evidence suggesting that the tetracyclines possess anti-apoptotic properties (Fig. 5). In a seminal study by Yrjanheikki et al. (101), it was found that MIN and DOX increased the survival of hippocampal neurons following global brain ischemia in gerbils, and this protection was associated with reduced caspase-1 expression. MIN was subsequently evaluated in several other models of neuronal injury and found to also be protective against Huntington's disease (8), traumatic brain injury (73), and Parkinson's disease (20). In each of these cases, the neuroprotection by MIN was associated with a reduction in caspase-1 and/or caspase-3 expression, suggesting MIN was protective by inhibiting the expression of key factors within the apoptotic cascade. In addition to inhibiting caspase expression, MIN has also been shown to inhibit caspase activity by blocking activation. Zhu et al. (103) demonstrated that MIN inhibits cytochrome c release and caspase-3 activation in mice with amyotrophic lateral sclerosis. Using isolated mitochondria, Zhu and colleagues also showed that MIN inhibited Ca^{2+}, and Bid induced mitochondrial swelling and cytochrome c release, indicating that the mitochondria, and perhaps the mPTP, were direct targets of MIN (103).In a follow-up study, the same group showed that MIN inhibited mitochondrial release of cytochrome c, Smac/DIABLO, and AIF in both a culture model and an in vivo mouse model of Huntington's disease, indicating MIN inhibits both caspase-dependent (cytochrome c and Smac/DIABLO) and caspase-independent AIF mitochondrial death pathways (95). MIN also protected cultured renal epithelial cells from I-R injury and inhibited mitochondrial cytochrome c release (92). Interestingly, this group found that protection by MIN was dependent on MIN-induced upregulation of Bcl-2, as antisense-mediated downregulation of Bcl-2 abolished the protective effects of MIN. Taken together, these studies demonstrate that MIN, and possibly also DOX, possess anti-apoptotic effects mediated by inhibition of caspase expression and by mitochondrial stabilization.

MIN has been shown to also improve myocyte survival and reduce infarct size, and this was correlated with reduced caspase expression and reduced mitochondrial release of cytochrome c and Smac/DIABLO (75). These studies demonstrate that blocking apoptosis by directly inhibiting factors within the apoptotic cascade can salvage myocytes and reduce infarct size. MIN has been shown to effectively block apoptosis in models of neuronal injury, and there is at least preliminary evidence that MIN can reduce infarct size by blocking apoptosis in the heart. Additional studies are needed to clarify this potentially important role of tetracyclines in the heart.

Anti-Inflammatory Effects of Tetracyclines

A class of compounds that can act as anti-proteolytic and ROS scavenger is likely to exert anti-inflammatory actions. It is well accepted in the dermatology field that rosacea is a disease of inflammatory nature (51). DOX are successfully used in the treatment of skin conditions such as acne and

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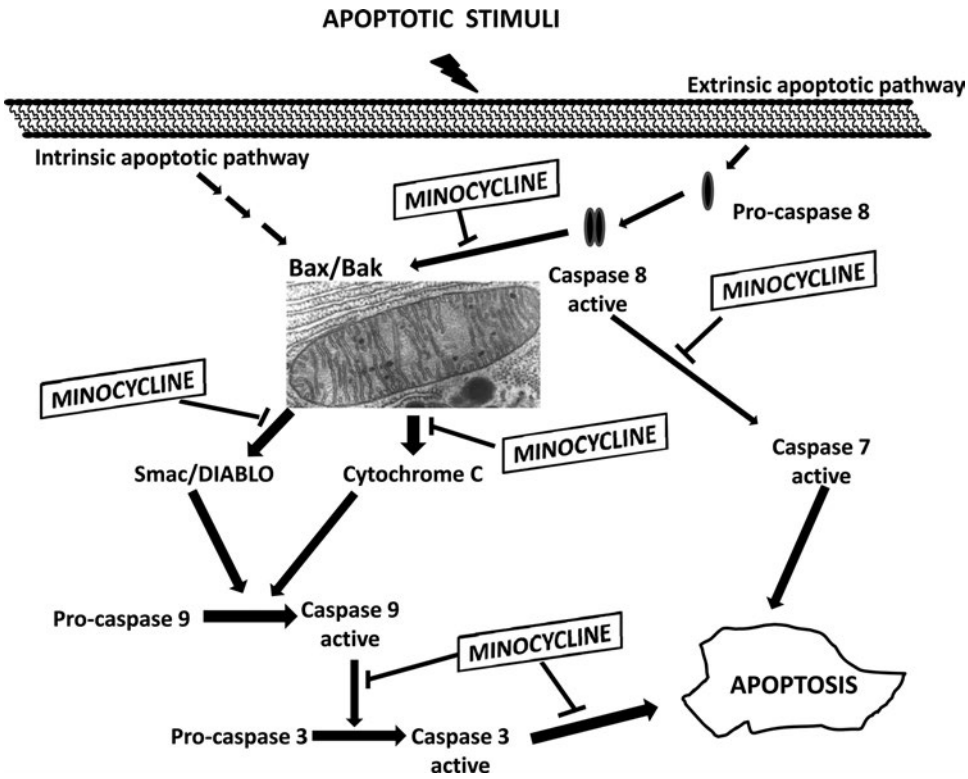


Fig. 5. Summarization of possible sites of action of minocycline on suppressing apoptotic signals in cells.

rosacea. Biopsies of inflammatory lesions of patients with acne yield increases in proinflammatory cytokines TNF-α and IL-1β (63). These cytokines are known inducers of increases in MMP levels and of their activity. ROS and nitric oxide (NO) have also been described as playing a role in the pathophysiology of rosacea (49). NO likely mediates increases in vessel permeability and edema and may support erythema development that occurs with rosacea. The beneficial effects of TTCs are likely associated with their aggregate "beneficial" actions including inhibiting proinflammatory cytokine levels, MMPs, and ROS. However, other actions add to their anti-inflammatory profile. MIN and to a lesser extent DOX can inhibit both pancreatic and nonpancreatic phospolipase A2 (65). This enzyme plays important roles in joint inflammation with a less clear participation in rosacea. The migration of white blood cells is critical for inflammation to occur. TTCs can inhibit neutrophil migration (22), adherence (26), and the proliferation of lymphocytes (87). Granuloma formation is a salient feature of many inflammatory diseases including rosacea. TTCs have been shown to inhibit granuloma formation of blood mononuclear cells in in vitro systems (996). Thus the cumulus of these features aggregates into the anti-inflammatory profile of TTCs.

Tetracycline Uptake by Tissues and Cells

Tetracyclines have been reported to concentrate at the site of tissue injury. In the 1970s investigators using radiolabeled tetracycline noted its capacity to accumulate in damaged myocardium and serve to diagnose infarcts (44, 45). Results demonstrated a correlation between infarct size, as determined by radiolabeled tetracycline, and serum creatine kinase. The ability of tetracycline to concentrate in other tissues is well known (2). Dentists take advantage of the high concentration of DOX (Periostat) in saliva as a means to treat gingivitis. To investigate tetracycline accumulation in gingival tissue, Yang et al. (98) examined the capacity of gingival fibroblasts to uptake the compounds. Gingival fibroblasts transport MIN in a concentration- and temperature-dependent manner. At steady state, the cellular/extracellular concentration ratio was >60 for MIN. The uptake of tetracyclines also has been observed in neutrophils and may partly explain high levels observed in injured tissues (91). Romero-Perez et al. (69) explored the capacity of MIN to accumulate in myocardial tissue and cells. MIN accumulated in myocardium severalfold greater than plasma levels. Accumulation was more pronounced in ischemic than normal myocardium. Cardiac fibroblasts and myocytes possess a comparable uptake system to that reported for gingival cells (98). Their intracellular concentration could in theory reach millimolar levels. At these concentrations mass action effects are likely seen where the drugs may at the same time exert potent antioxidant, anti-MMP, and other effects that in balance ultimately translate into cytoprotective effects.

Other Potential Uses of Tetracyclines

The pleiotropic properties of tetracyclines are not necessarily limited to those described above. Indeed, this class of drugs have been reported as exerting unique effects on complex pathologies. In an experimental simian immunodeficiency virus (SIV) model of HIV central nervous system (CNS) disease, MIN reduced the severity of encephalitis, suppressed viral load in the brain, and decreased the expression of CNS inflammatory markers (102). Tetracyclines also demonstrate protective effects on prion [PrP(Sc)]-mediated brain damage. In one study Syrian hamsters were injected intracerebrally with scrapie-infected brain homogenate that was coincubated with 1 mM tetracycline before inoculation (25). Animals showed a significant delay in the onset of clinical signs of disease and

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prolonged survival time. Effects were paralleled by a delay in the appearance of magnetic-resonance abnormalities in the thalamus, neuropathological changes, and PrP(Sc) accumulation. When tetracycline was preincubated with highly diluted scrapie-infected inoculum, one third of hamsters did not develop disease. Thus tetracyclines appear to reduce prion infectivity through a direct interaction with PrP(Sc) and are potentially useful for inactivation of bovine spongiform encephalopathy (BSE)- or variant Creutzfeldt-Jacob disease-contaminated products and prevention strategies.

There are few agents as acutely damaging to tissues and living organisms as mustard gas. This alkylating agent causes massive blistering of the skin and severely damages the lungs by activating proteases (including elastases and MMPs) among other effects. In a study by Guingabert et al. (38) guinea pigs were given mustard gas intratracheally (38). A group of animals were pretreated with DOX resulting in decreased gelatinase activity, decreased inflammation, and notable decrease in histological lung epithelial lesions. Acute respiratory distress syndrome (ARDS) develops in the setting of diseases such as sepsis. With ARDS an infiltration of the lungs by neutrophils can lead to a massive activation of the cells yielding local tissue destruction and possibly the death of the subject. The destruction of lung tissue can be documented in bronchial lavage by the presence of protease such as elastases, MMP, collagen, and elastin fragments. In a study by Steinberg et al. (83) the prophylactic use of the CMT COL-3 was examined in a chronic insidious onset animal model of sepsis-induced ARDS. COL-3 prevented the development of ARDS and also prevented septic shock. The emergence of new epidemics where ARDS may be an important component to the cause of severe disease or death raises the spectrum of the possible use of tetracyclines to prevent or limit the development of respiratory system complications. Such is the case for H1N1 influenza and SARS (severe acute respiratory distress syndrome caused by a coronavirus). A potential scenario may be where there is high risk of exposure and contamination by a mutated version of a virus (such as in the case of the H1N1 influenza) by medical personnel where the use of tetracyclines may be justified as a means of preventing serious complications from developing upon exposure and infection.

CMT (in particular COL-3) have also been examined for their anti-cancer therapeutic potential. MMP are involved in tumor metastasis and angiogenesis and are overexpressed in Kaposi's sarcoma (KS) cells. In a phase II study, there were significant declines in MMP-2 and MMP-9 plasma levels from baseline to minimum value with treatment, and the most common adverse events were photosensitivity and rash. COL-3, when administered at 50 mg/day, demonstrated antitumor activity similar to other promising investigational KS drugs.

Ongoing Clinical Trials

Table 1 summarizes the most relevant clinical trials implemented (excluding those associated with their antibiotic or dermatologic effects). As can be ascertained from the list, this class of drugs is receiving a great amount of attention pertaining to their apparent capacity to beneficially modulate various apparently unrelated diseases. The table also includes (in very general terms) the apparent cause associated with a particular disease where tetracyclines are being tested.

Table 1. *Most relevant clinical trials implemented using minocycline, doxycycline, and chemically modified tetracycline COL-3*

Disease	Cause
Minocycline	
Acute kidney injury	Ischemia
Acute spinal cord injury	Ischemia
Amyothrophic lateral sclerosis	Autoinmmune
Asthma	Immune - inflammation
Autism	Unknown
Cognitive function/endarterectomy	Ischemia
Dry eye syndrome	Inflammation
Fragile X syndrome	Hereditary mental retardation
Gum disease	Inflammation
HIV – cognitive impairment	Viral - inflammation
Huntington's disease	Degenerative
Idiopathic pulmonary fibrosis	Unknown - inflammation
Ischemic stroke	Ischemia
Multisystem atrophy	Degenerative
Parkison's disease	Degenerative
Pneumothorax	Inflammation
Psychosis	Unknown
Rheumatoid arthritis	Inflammation
Schizophrenia	Unknown
Sclerosis cholangitis	Inflammation
Doxycycline	
Abdominal aneurysm	Inflammation - proteases
Alzheimer's disease	Degenerative
Bone loss	Hormonal imbalance
Brain hemorrhage	Ischemia
COPD	Inflammation
Coronary artery bypass	Ischemia
Diabetic retinopathy	Inflammation
Diabetic ulcers	Inflammation - healing
Lymphangioleiomyomatosis	Noncancerous tumor
Osteoarthritis	Inflammation
Postmyocardial infarction remodeling	Inflammation - healing
COL-3	
Metastatic cancer	Cancer
Kaposi's sarcoma	Cancer
Recurring brain tumors	Cancer
Advanced solid tumors	Cancer

Partial listing of clinical trials in different phases of execution as gathered from www.clinicaltrials.gov for minocycline (20/54 total), doxycycline (11/88), and the chemically modified tetracycline COL-3 (4/4). The listing only includes nonconventional uses.

Conclusions

Tetracyclines have been recognized slowly over time as a genre of drugs with interesting pleiotropic properties. Their accumulation in injured tissues makes them almost appear to act as a smart drug. The recognition by scientists and clinicians of these collection of properties and of the safety profile of this class of drugs has led to the implementation of clinical trials to explore their possible beneficial effects in the setting of a wide variety of diseases. Clinical trial efforts so far have been mostly isolated but, as a whole they would appear to require the formation of a consensus group that would lead to a more systematic understanding (and examination) of their therapeutic potential. As CMT have proven to generate useful derivatives with unique properties, a rational modification of this class of drugs may lead to the development of novel compounds with greater therapeutic potential and safety profiles.

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Indeed, such creative efforts are currently the focus of various research groups (84). However, it should be recognized that each member of the TTC family has both similar and more importantly, distinct properties from each other such as half life and lipophilicity. Scientists when anticipating their use, need to make extensive considerations towards the desired "preferred" action (e.g., antiapoptotic vs. antiprotease) and anticipated outcomes.

GRANTS

This work was supported by a NIH HL-43617 and AT-004277 to F. Villarreal, and G. Ceballos by a visiting professor CONACYT fellowship from Mexico.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Inflammation Research (2021) 70:847–858
https://doi.org/10.1007/s00011-021-01487-6

FOI 26-3132 - Document 5

Inflammation Research

REVIEW

SARS-CoV-2 and pathological matrix remodeling mediators

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Received: 7 April 2021 / Revised: 4 July 2021 / Accepted: 7 July 2021 / Published online: 20 July 2021
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Abstract

Background Recognizing only sharp elevation in a short period of time, the COVID-19 SARS-CoV-2 propagation is more and more marked in the whole world. Induced inflammation afterwards infection engenders a high infiltration of immune cells and cytokines that triggers matrix metalloproteinases (MMPs) activation. These endopeptidases are mediators of the lung extracellular matrix (ECM), a basic element for alveoli structure and gas exchange.

Methods When immune cells, MMPs, secreted cytokines and several other mediators are gathered a pathological matrix remodeling occurs. This phenomenon tends to tissue destruction in the first place and a pulmonary hypertrophy and fibrosis in the second place.

Findings After pathological matrix remodeling establishment, pathological diseases take place even after infection state. Since post COVID-19 pulmonary fibrosis is an emerging complication of the disease, there is an urge to better understand and characterize the implication of ECM remodeling during SARS-CoV-2 infection.

Conclusion Targeting MMPs and their inhibitors could be a probable solution for occurred events since there are many cured patients that remain with severe sequels even after the end of infection.

Keywords MMPs · Matrix remodeling · Immune cells · Cytokine · SARS-CoV-2 · Therapeutic target

Introduction

The emerging pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) brought a state of alert around the world. SARS-CoV-2 belongs to the *Betacoronavirus* family which includes two other viruses known for other pandemic diseases: the Middle East Respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV), respectively [1–4]. SARS-CoV-2 shares 79.6% of its genome with SARS-CoV-1 and 96% with the bat coronavirus (RaTG13) which make it most likely deriving from the later [1, 5]. SARS-CoV-2 shares important sequence homology with SARS-CoV but also a common entry receptor angiotensin-converting enzyme 2 (ACE2). For such reason, several authors have suggested that their biological properties could be similar and understanding host response to SARS-CoV-1 may help us understanding SARS-CoV-2 implication at various levels [4, 6, 7].

Latest models suggest that following SARS-CoV-2 invasion in type II pneumocytes (via ACE2) leads to a rapid virus replication as well as concomitant intense secretion of pro-inflammatory cytokines, chemokines, and other proteases are mainly secreted by the innate immune cells' response [8]. Subsequent excess inflammation, especially in the lung further (the so-called "cytokine storm") leads to acute respiratory distress syndrome (ARDS), pulmonary edema and endothelial damage [1].

On a molecular scale, when all of these inflammatory mediators are gathered, there is an increase of matrix metalloprotease (MMPs) secretion in site of the inflammation. This increase leads to a pathological remodeling of lung's extracellular matrix (ECM) [1, 8–10]. MMPs are members of the proteolytic zinc-dependent family. There are 24 recognized members until today and they are included in several vital mechanisms and pathways including majorly wound healing, ECM degradation, angiogenesis, proliferation and

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FOI 26-3132 - Document 5

signaling pathways [11, 12]. While present in low quantity at homeostasis, their increased secretion and imbalance have been widely studied in various lung pathologies in chronic and acute nature.

Since they are involved in different pathways and physiological phenomena, disequilibrium in their levels opens the door to pathological situations translated by serious diseases like cancer, cardiovascular diseases, neurodegenerative diseases and chronic inflammation which explains why they drew so much attention lately. Even though the fact that they are capable of modulating cellular behavior in a non-specific way, they could be considered as therapeutic targets [13]. Secreted MMPs enhance pro-inflammatory cytokines, bioactive chemokines and oxygen reactive. They are even included in infection propagation in the organism through their members with transmembrane domain like ADAM-17 which leads us to ask about their real implication even with fluctuated levels [14–16]. In fact, metallopeptidases are probably included in immune cells infiltration since they are mediators of ECM and basement membrane breakdown that surrounds the parenchymal ECM and blood vessels.

The combination of ARDS and hyper-inflammation has the potential of driving pulmonary fibrosis. Since post-COVID-19 pulmonary fibrosis is an emerging complication of the disease, there is an urge to better understand and characterize the implication of ECM remodeling during SARS-CoV-2 infection. This review focuses on current and emerging evidence regarding the pathophysiological role of metallo-endopeptidases, particularly MMPs, in determining the remodeling process in lung tissue damage during SARS-CoV-2 infection.

The SARS-COV-2/ACE2 pathway to infection

Through genome sequencing and by homology modeling, the angiotensin-converting enzyme 2 (ACE2) has been reported to be the target for SARS-CoV-2 [3]. Specific binding between spike (S) glycoprotein of coronavirus and the ACE2 receptor followed by S cleavage by serine protease 2 (TMPRSS2) allows for virus entry to cell [1, 4, 17]. Several studies of gene expression datasets have reported that ACE2 more than TMPRSS2 may be the limiting factor for viral entry at the initial stage [3]. Moreover, the study reported a high expression of ACE2 in nasal secretory and ciliated cells as well as type II alveolar epithelial cells (AEC). At homeostasis, quiescent types II AEC are the major source of ACE2 within the lung. During lung fibrosis, those cells actively proliferate and downregulate the expression of this enzyme. ACE2 expression is severely downregulated in the context of pneumocyte proliferation and lung fibrosis [18].

The viral spike protein (~ 1300 amino acid residues) is formed by two functionally subunits: S1 contains the receptor-binding domain (RBD) attached to S2, which contains the post-fusion core [19, 20]. This process is crucial for the virus cell entry resulting in shedding S1 subunit from the viral surface [4]. The S-protein undergoes conformational rearrangements allowing the S2 subunit a post-fusion conformation [19, 21, 22]. Besides the proteolytic cleavage of SARS-CoV-2 S by TMPRSS2, a smaller rate of endosomal cysteine proteases cathepsin B (CatB) and cathepsin L (CatL) has been reported to assist in priming and entry process [4, 23] (Fig. 1A). A distinctive feature of the SARS-CoV-2 S-protein is the Furin cleavage site acquirement, polybasic cleavage site (RRAR) at S1–S2 junction which is cleaved efficiently by furin enzyme [24]. The S1/S2 junction and S2′ are the two cleavage sites in viral S-protein, allowing the activation and priming process through host cell enzymes trypsin, cathepsins, furin and TMPRSS2 [4, 25]. After this bond, the virus membrane and the host cell membrane fuse together through their ligand–receptor link and then the virus enters the host cell [26]. The angiotensin-converting enzyme 2 (ACE2) is a homolog of the ACE.

ACE2 is a widely expressed enzyme with pleiotropic roles which is part of the Renin–Angiotensin System (RAS). ACE2 is the homologous of ACE and shares a high affinity to the angiotensin II (ANG-II) [6, 27, 28, 31, 32]. There are three discovered receptors for the ANG-II until today. Among these receptors, the angiotensin I (AT1R) leads to vasoconstriction, induces inflammatory responses and enhances matrix remodeling, whereas the AT2R reverses the ANG-II effects [28, 29], while ACE–ANG-II–AT1R axis activation has been proven to causes deleterious effects, such as vasoconstriction, inflammation, and fibrosis [28–30]. The ACE2–ANG I–7–Mas axis appears to counter-regulate the ACE–ANG-II–AT1 negative effects anti-thrombotic, anti-proliferative, anti-fibrotic, and anti-inflammatory properties [31, 32].

ACE2 acts like a transmembrane enzyme with an extracellular domain and presents a target site for the SARS-CoV-2 to mediate its actions [33]. When the SARS-CoV-2 enters into the organism, it binds directly through its glycoprotein spike existent on its viral membrane to the host cell receptors [4, 34]. The recently made study by Zhou and its collaborators demonstrated that SARS-CoV-2 entry to the host cell is mainly insured by the ACE2 cell receptor since cells that do not express this receptor are not infected [1]. However, on T lymphocytes in vitro, SARS-CoV-2 is capable of using other cellular receptors of S-protein to infect cells that do not express the ACE2 receptor [35]. It is plausible that interaction of ACE2 with SARS-COV-2 may induce its downregulation and thereby enhance the ACE-AngII-AT1 axis leading to vasoconstriction, pro-inflammatory and oxidative stress. In a recent study, Liu et al. reported higher serum Ang II levels in patients with higher viral load which further supports this hypothesis [36]. Afterwards, the viral RNA is injected into

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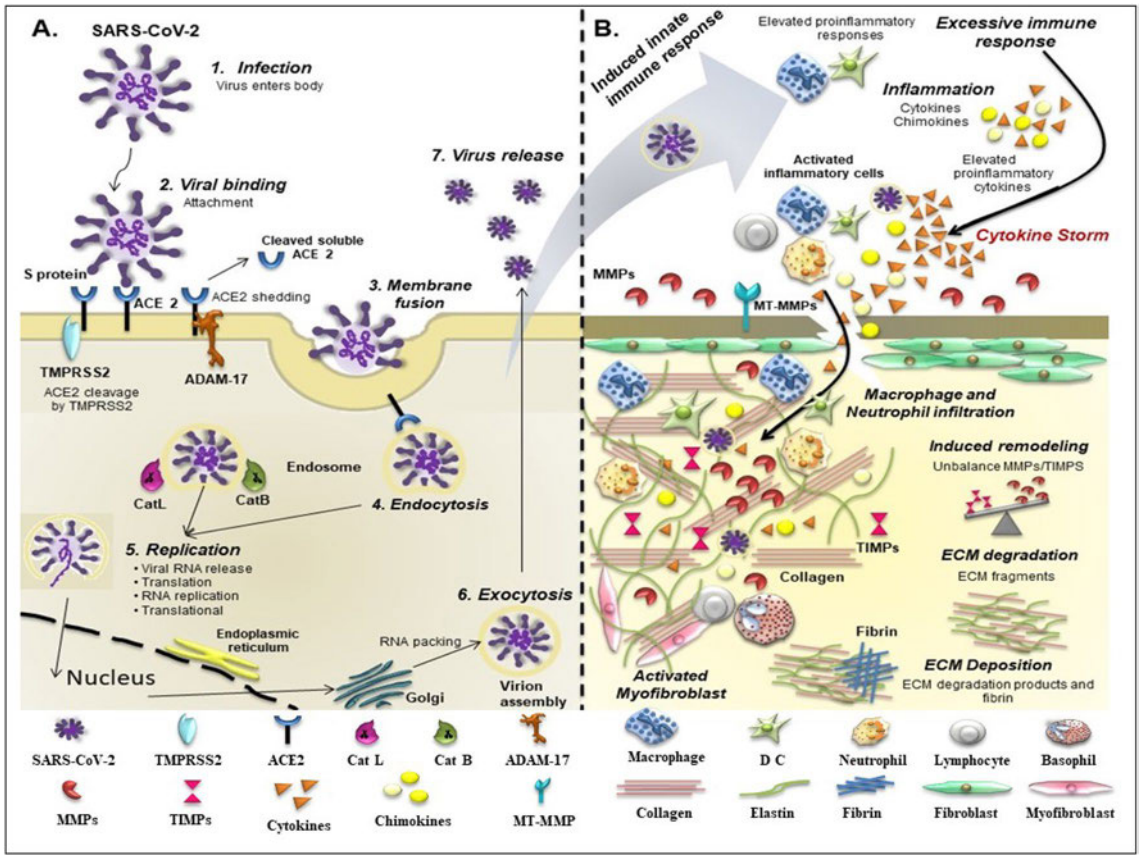


Fig. 1 Induced pathological matrix remodeling after SARS-CoV-2 infection. **A** Cell entry mechanism of SARS-CoV-2 summary: the initial step of SARS-CoV-2 infection involves a specific binding of S-protein to host cell entry receptors, angiotensin-converting enzyme 2 (ACE2). Gathered together and with the host cell surface serine protease TMPRSS2, the entry process is activated. Following the endocytosis, cathepsins are released. These proteases modify once again the S-protein and promote the release of viral RNA into the cytoplasm. **B** SARS-CoV-2 Physio-pathological implications: when virus propagation occurs, a massive destruction of tissues takes place. An epithelial damage is the initial hallmark of SARS-CoV-2 infection. A specific immune response is induced to eliminate the virus and initiate the inflammatory response. The immune cells, in particular, macrophages identify the virus and release pro-inflammatory mediators (cytokines, chemokines, growth factors…). Cytokines activate more immune cells, which in turn produce more cytokines creating an uncontrolled cycle of inflammatory response "cytokine storm". Activated immune and non-immune cells produce MMPs which lead to ECM breakdown disruption and this unresolved infection gives rise to damages. These damages do happen during the cytokine storm which includes the unbalance of the MMPs/TIMPs ratio, the increase deposition of ECM fragments, and the massive formation and deposition of fibrin. *ACE2* angiotensin-converting enzyme 2, *ADAM-17* ADAM metalloprotease domain 17, *Cat L* cathepsin L, *Cat B* cathepsin B, *TMPRSS2* transmembrane protease serine 2, *DC* dendritic cell, *MMPs* matrix metalloproteinases, *MT-MMPs* membrane-type matrix metalloproteinases, *TIMPs* tissue inhibitors of matrix metalloproteinases

the cell cytoplasm, and its open reading fragment (ORF) is directly translated into proteins. These proteins are cleaved by their viral proteases generating their RNA-dependent RNA polymerase (RdRp) necessary for RNA replication. Genomic RNA is thereby replicated by its specific RdRp in presence of structural proteins (Spike, envelop protein, Membran Protein and Nucleocapsid) and is translated by the Golgi complex and the endoplasmic reticulum's host cell. The replicated RNA and structural proteins are gathered to produce new viral particles and are secreted by exocytosis [6, 7] (Fig. 1A).

FOI 26-3132 - Document 5

From lung infection to extracellular matrix remodeling

A very thin alveolocapillary membrane allows oxygen and CO_2 to diffuse through the cells. During the first level of infection, the viral spike binds to the ACE2 receptor on the host cell surface. This bond leads to the incapacity of regulating ANG-II which provokes a sudden increase of the inflammatory response. This increase induces the activation of a large scale of cells including cytokines, and MMPs [37–39]. The sudden activation of these endopeptidases induces matrix disequilibrium and this phenomenon leads in turn to the collapse of matrix. On the one hand, MMPs will be highly activated since the inflammatory response implies a storm of cytokines. On the other hand, the ANG-II increase leads to MMPs increase. In a study made on tuberculosis pleural effusion, ACE2 and MMP-9 do correlate negatively, more precisely when ACE2 receptors are taken by the virus, an increase in ANG-II arises leading to the MMP-9 activation [40]. ACE2 is a pleotrope receptor; it counterattacks the ANG-II and leads to vascular wall hypertrophy. This receptor captures the ANG-II and converts it to reverse its effect. During the infection, the receptor is linked to the virus for its profit. In response to the infection, a lung injury and organ failure occur. As SARS-CoV-2 emerges in human body, a huge storm of inflammatory cytokines comes by. Tissue damage is specific to the virus and then it also appears in multiple other damages [39]. Induced proteolysis by secreted MMPs through the activated inflammatory cells and cytokines has been included in a fatal process. MMPs' proteolytic action seems to be involved in COV-2 pathogenesis, similar to several diseases that are related to tissue damage (Fig. 1B).

The role and fate of the pulmonary extracellular matrix

The lung has a very specific ECM. At healthy state, the ECM forms a thin membrane which allows for passive diffusion of passive diffusion of gas through alveolar-interstitial membrane. The alveoli air exchange structure is composed mainly of an air barrier. This barrier comprises a capillary and an alveolar epithelium, an extracellular matrix and alveolar mediators including fibroblasts and macrophages [41]. To facilitate ventilation, this structure maintains in the same time gas exchange and ECM mechanical properties. ECM is also implicated in inter-cell signaling, in cell function, proliferation and shape. It is highly sensitive to growth factors, such as TGF-Beta. Type I alveolar cell ensures the gas exchange through basal lamina. As for the rest of the solute and liquid exchange, it is ensured by ECM components [41, 42]. During fibrosis, the ECM expands which limits gas diffusion. Also, a pathological fibro-proliferation of the alveoli ECM arises [43, 44]. Lung ECM is a complex and dynamic tissue that has a tremendous role in maintaining a normal connective structure. In lungs, ECM is mainly composed of a thin basement membrane and an interstitial tissue that forms the lung's parenchyma [45] and provides structural support for gas exchange by developing a branched architecture [46]. Also, ECM is widely implicated in signaling pathways through its specific mediators, in cell function and shape, in proliferation mechanisms and in responding to growth factors [47–49]. Lung ECM major components are collagen and elastin fiber that are overlapping with glycosaminoglycans, proteoglycans, fibronectin fibrils and sequestered water [46] (Fig. 2A). Resident fibroblasts are abundant and are mainly in charge of ECM production. Furthermore, laminin, heparan sulfate, hyaluronate, nidogen/entactin, and matricellular proteins like thrombospondin, tenascin C, and tenascin-X have been also identified in alveolar tissues. With all of this wealth in the matrix structure, collagen is the most abundant protein in lung tissue. In lung parenchyma, collagen types I and III predominate the alveolar membrane [46, 50]. After SARS-CoV-2 infiltration in lungs, an acute severe inflammatory response occurs. Following viral entry into host cell, RNA is recognized by the cytoplasmic pattern recognition receptor (PRR) leading to transcription factors activation through the toll interleukin receptor 1 containing interferon beta (TRIF). This activation leads to interferon-γ (IFN-γ) secretion [51, 52]. However, an insufficient level of IFN-1 for infection control enhances viral replication. In other patients, IFN-γ serum level is more important. This fact is the reason behind the cytokine storm particularly myeloid cytokine (IFN-γ; GM-CSF). Their activation attracts immune cells (monocytes, lymphocytes, peripheral blood mononuclear cells) in the infection site [53].

IFN-γ production during early infection phase is the immune defense key against the virus emerge [51] and a delay in their release at early stages of COVID-19 makes it harder for the antiviral response. This release enhances cytokine secretion and through the chemiotactic phenomena, more inflammatory cells are attracted. Afterwards, an extreme infiltration in lung tissue arises [54, 55]. The emerging concept elaborated by McGonagle and his collaborators discussed the probability that the induced cytokine storm during SARS-CoV-2 infection result from the immune system failure to eradicate the virus. They divided the storm in two phases [53]. At a first level, there is a loss of the first anti-viral line defense. In fact, the Toll receptor like-3 (TLR-3) is capable of recognizing the double-stranded viral RNA. This fact leads to TRIF activation which in turn activates Interferon Regulated Factor 3 (IRF-3) and Nuclear Factor-kappa B (NF-κB) to enhance IFN-γ and Tumor Nescrosis Factor-β (TNF-β) production. Theses pro-inflammatory

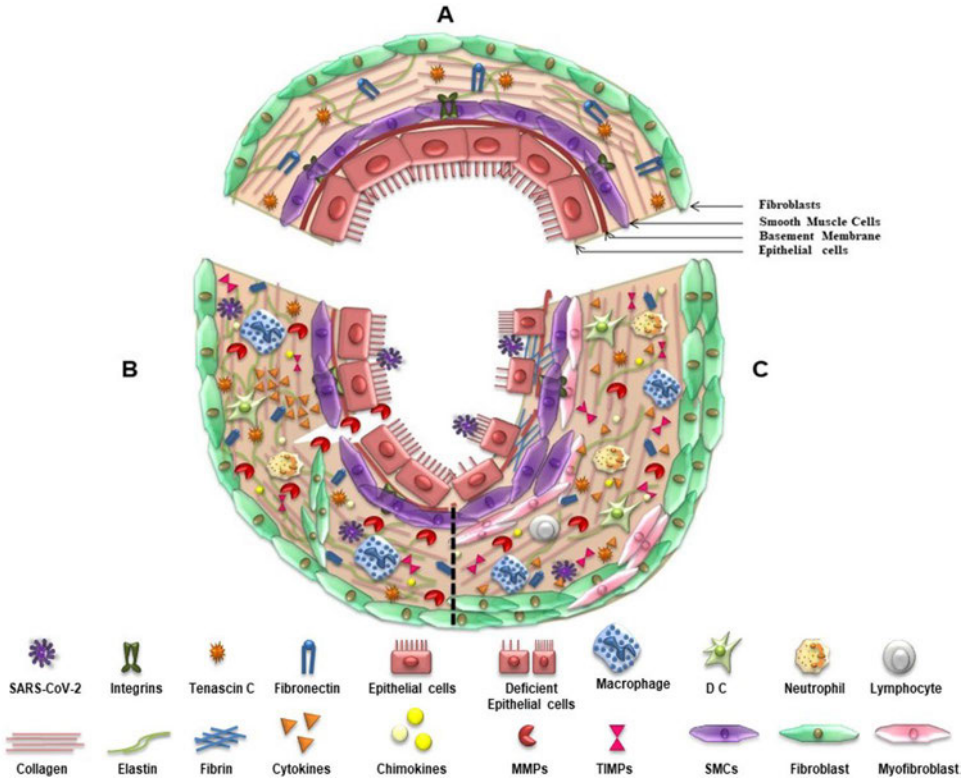


Fig. 2 ECM lung evolution from healthy to a pathological matrix. **A** Healthy lung matrix structure is preserved by the presence of fibroblasts, collagen, elastin and anchored fibronectin to basal membrane. **B** ECM breakdown after virus infiltration and MMP overexpression (from day 1 to 5 of infection): collagen fibers destruction through secreted MMPs by inflammatory cells. This destruction leads to basement membrane disruption. **C** ECM lungs fibrosis and hypertrophy (from day 5 of infection and more): after increased degradation, a remodeling takes place to respond to lung injuries. However, ECM mediators' imbalance and SMC migration lead to increased collagens and matrix degradation products deposition that are presumed in a thickened matrix. *SARS-CoV-2* severe acute respiratory syndrome coronavirus-2, *MMP* matrix metalloproteinase, *TIMP* tissue inhibitor of matrix metalloproteinase, *ECM* extracellular matrix, *SMC* smooth muscle cells, *DC* dendritic cells

cytokines are considered to be in the first line of defense and the loss of this is due to the viral charge and hypo-secretion of IFN-1 [51, 53, 55].

As a consequence, a second storm is activated, that is more tissue aggressive than the first one to cover up for the first target failure during the first response [55, 56]. During this phase, myeloid IFN-γ pathway and derived cytokine that includes TNF, GM-CSF and IL-1 are overexpressed in blood, rendering it in a hyper-cytokinaemic mode. The cytokine shock is explained until this day as the ultimate uncontrolled systemic inflammation that is triggered by cytokines hypersecretion after infection which leads to organ failure and that might cause death [57].

When all of these factors are gathered all together, a tissue destruction could occur. In fact, following the viral infection by the SARS-CoV-2 virus, an ECM breakdown takes place which leads to the aggravation of lung state and provokes lung pathologies progression (Fig. 2B). The immune cells influx into lungs does protect the host cell against excessive immune responses including both innate and adaptive. This fact enhances chronic inflammation which in turn destroys the lung's parenchyma [58, 59]. T lymphocytes, the adaptive immune cells, are headed to ECM lung's components. These mediators are capable of inducing abnormal immune responses that enhance lung destruction. During innate and adaptive immunity activation, triggered immune cells induced innate inflammation in the lung. Excessive immune response starts a sequence of signals that overlaps lung stroma which are exposed to antigen-presenting cells (APCs) towards lymphocytes for generating the major histocompatibility complex (MHC) [60]. To activate APCs, specific signals must be secreted. ECM breakdown and degradation products may engender new antigens that are recognized by immune cells resident in lung [60–63]. This injury engenders an alveolar basement membrane disruption and the accumulation of matrix degradation products that are rich in fibrin [64]. ECM bioactive fragments are in turn mediators of inflammation since collagen degradation is implicated in neutrophil migration. Collagen I Proteolytic

FOI 26-3132 - Document 5

fragment is capable of acting like chemokines and attracts immune cells by binding to their receptors [65, 66].

Lung ECM major components were absent in infected lungs with SARS-CoV-2 which leads to a severe mechanical damage presumed in COVID-19 characteristics [67]. In their recent proteomic analysis of lung tissues from deceased patients suffering from severe forms of COVID-19, Leng et al. reported several major components of ECM homeostasis to be downregulated. For example, the ECM protein annexin A2 (ANXA2), which regulates surfactant secretion by type II pneumocyte was significantly downregulated [67]. Surfactant dysregulation plays a major role in ARDS pathophysiology [68]. Thereby, maintaining a normal state of surfactants could improve the prognosis of severe patients suffering from COVID-19 [69]. ECM fate is related to a balance between degradation and secretion of matrix components. It is related to myofibroblasts after injury for a fibrotic repair since interactions between fibroblasts and ECM components enhance matrix production and stimulate signaling pathways [35, 70] (Fig. 2C).

Mediators of lung ECM breakdown

ECM breakdown is mediated by MMPs that are capable of degrading ECM components and are characterized by the presence of the metallic ion Zn^{2+} that confers the catalytic activity [11]. There are 28 MMPs discovered in vertebrates until this day [71], 24 of which are expressed in human tissues; they are divided according to their structure and their substrate specificity [72]. Their basic structure is limited to a pre-domain which represents a signal peptide for secretion and is eliminated after its secretion. The pre-domain is followed by a pro-domain that ensures the enzyme latent form and is mainly formed by a peptide sequence containing a cysteine residue which interacts with the catalytic site. This site has a zinc atom maintained by three histidine residues in a tri-dimensional structure [11, 73]. Thereby comes the catalytic domain which comprises a linked site to zinc with a highly conserved sequence *HEXXHXXGXXH* [74]. Pro-domain cleavage releases the zinc ion and activates the catalytic site that can bind afterwards to its substrate through H_2O penetration.

According to their specificity and primary structure, the first group is composed of collagenases (MMP-1, MMP-8 and MMP-13). The second group englobes the gelatinases (MMP-2 and MMP-9) and the third the Stromelysines (MMP-3, 10 and 11). MMPs that contain a membrane-type (MT-MMPs) are members of the fourth group that can be subdivided into two subgroups, type I transmembrane proteins (MMP-14, 15, 16, and 24) and glycosylphosphatidylinositol (GPI) anchored proteins (MMP-17 and -25) [75]. The enzyme structure is also formed by a hinge region and a hemopexin domain that interferes into MMPs recognition of their substrate except for MMP-7, 23 and 26 that do not possess the hemopexin domain [13, 73, 76]. Secreted MMPs at site of lung inflammation are capable of modulating inflammatory mechanisms whether by affecting chemokine activity, disposal or by targeting proteins that are in a direct association with their activity [77–79].

As for adamalysins, they are in the same time metalloproteinase and disinegrin and are characterized by a thrombospondin motif [80]. Their structure is mainly composed of a pro-domain, a zinc-binding domain, a disintegrin domain that confers interaction to the ADAM, a cysteine-rich domain for ECM interaction, an epidermal growth factor domain and a cytoplasmic tail [81, 82]. Also, the fact that ACE2 contains a HEXXH zinc-binding motif makes it a metalloproteinase [33]. MMPs are capable of modulating inflammatory mechanisms whether by affecting chemokine activity, disposal or by targeting proteins that are in a direct association with their activity [77, 78].

Secreted MMPs by macrophages like MMP-12 and MMP-28 are included in the pro-inflammatory process by enhancing macrophage infiltration in lungs [83]. Another mediator of immune cells is the matrilysin (MMP-7). This MMP does promote neutrophil infiltration via the epithelial barrier by degrading the transmembrane proteoglycan coordinated to the KC. The KC is a murine chemokine (CXC) that is implicated in attracting inflammatory cells [77]. The MMP-8 nearly shares this characteristic from the MMP-7 and possesses a chemiotactic effect on neutrophils but in a more active structure. Under the influence of chemokines, neutrophils secrete MMP-8 which in turn facilitates neutrophils efflux through degrading the ECM in a feedback way. In the same meaning, gelatinase A and B (MMP-2 and MMP-9) interfere with recruited immune cells from peribroncho-vascular in alveolar space through modulating chemokines [84, 85]. A recently made study showed that MMP-9 level was higher in first 3–5 days of infection. This elevation correlated negatively to the arterial partial pressure of oxygen/fraction of inspired oxygen ratio (P/F) which means sicker patients [86].

MMP-9 has been related to lung diseases through tissue remodeling like acute lung injuries asthma pulmonary fibrosis and pleural effusion. Released MMP-9 from inflammatory cells does promote alveolar capillary destruction leading to lung injury [83, 84, 87]. Also, Shi et al. suggested that MMP-3 serum level may be a valuable marker of COVID-19 by indicating a significant difference found between novel coronavirus pneumonia-infected patients and healthy people [88]. This particular MMP is widely expressed in human tissues since it is capable of cleaving several components of ECM components. Its main role is the ability to activate other pro-MMPs. During matrix turnover, MMP-3 or stromelysin-1 activates matrilysin, collagenases, gelatinase

FOI 26-3132 - Document 5

B and pro-MMP-1 to generate an active form of MMP-1. Its capacity of cleaving type I collagen makes it different from collagenase [13, 89].

Following the emerging cytokines flow after infection, tissue inhibitors of metalloproteinase (TIMPs) are secreted to maintain the matrix equilibrium and to regulate cytokines shedding, playing therefore a tremendous role after injury caused mainly by inflammation [90–93]. The TIMP-3 is a specific inhibitor of MMPs and ADAM family (metalloproteinase and disintegrin) and is expressed by several cell types via many tissues. This inhibitor is capable of inhibiting the very particular ADAM-17 [94–96], a metalloprotease and a disintegrin that cleave not only tumor necrosis factor alpha (pro-TNF-alpha) [97, 98], but also responsible of shedding the ACE2 receptor and seem to be implicated in the propagation and the aggravation of the infection. Binding SARS-CoV-2 to the ACE2 induces its shedding by ADAM-17, and its uptake by the virus in turn [14–16]. This shedding enhances probably several cellular signals including PKC activation [15] and is involved in virus uptake towards target cells [95]. The emergent hypothesis is that ACE2 down-regulation by ADAM-17 could be responsible for lung tissue damage and respiratory failure aggravation by the virus. It has been reported, in vivo and in vitro, that the inhibition of ADAM-17/TACE blocks ACE2 shedding and limits viral entry [15, 16]. Its exact role in SARS-COV-2 invasion needs further investigations, indeed, while ADAM17 ability to cleave ACE2 is undisputable, it was demonstrated that only cleavage by TMPRSS2, and not by ADAM17 can promote virus entry into the cells in a SARS-CoV-1 model [95]. To more understand SARS-CoV-2 implication, we went through other possible pathways on viruses that belong to the same family. On an induced MHV-A59-infected mice model (MHV is a mice hepatitis virus that belongs to the same family of Beta-coronaviruses, where SARS-CoV-2 is a considered member of this family) [17, 99–103], there was an upregulation in MMP-3, MMP-12 transcript levels. There was also an elevation in TIMP-1 circulating levels and mRNA [101]. This inhibitor is found to be correlated with viral virulence and suggests a probable implication in the antiviral response [101]. Since Beta-coronaviruses entry depends on cellular protease for a successful penetration in host cell, there is a probable downregulation of TIMP-2, TIMP-3 and TIMP-4 [92, 104, 105]. In fact, basic TIMP-1 levels are in first place low but are highly induced after triggered immune response on the contrary of TIMP-2, TIMP-3 and TIMP-4 that are constitutively secreted and modifications in their levels are lower than those of TIMP-1 in immune response [105–109]. The increase of MMP-3 [88] and MMP-9 [85] circulating levels during first days of infection on the optimum of the viral charge and a probable downregulation

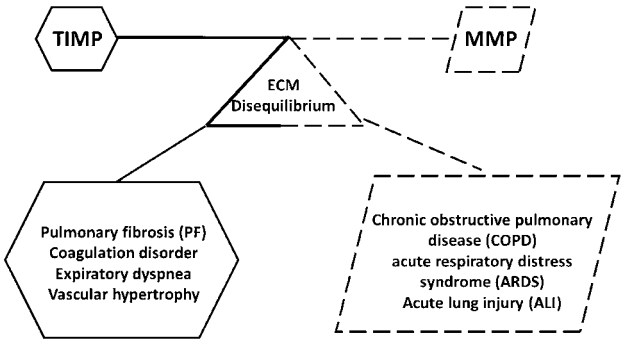


Fig. 3 Targeting ECM mediators as a therapeutic solution after SARS-CoV-2 infection could prevent from severe complications: matrix equilibrium is primordial for maintaining a healthy lung structure, when MMP/TIMP ration is in favor of MMPs, a matrix destruction occurs and when it is in favor of TIMPs a thickened matrix is observed. *MMP* matrix metalloproteinase, *TIMP* tissue inhibitor of matrix metalloproteinase, *ECM* extracellular matrix

of their specific inhibitors could put the puzzle in this way: the virus turns all of the mediators in its favor by enhancing MMPs secretion during first days of infection that triggers a matrix disequilibrium. Their decrease in the upcoming period suggests an upregulation of their specific inhibitors to respond their sudden elevation. More precise experiments, subsequent measurements and longitudinal analyses could better explain this phenomenon.

Are MMPs and their inhibitors probable therapeutic targets?

An elevation in MMPs level implies a matrix disequilibrium that will probably modulate in turn their specific inhibitors (Fig. 3). These inhibitors are addressed towards MMPs to regulate their action. TIMPs used in lung injury mice models reduced the inflammatory state and ameliorated the injuries [109]. Induced inflammatory markers during the cytokine storm, enhance TIMPs secretion particularly TIMP-3 since it is mainly secreted by macrophages [110]. TIMP-3 is the highest ECM regulator because of its capacity to bind directly to the matrix [111]. Capable of shedding receptors and cleaving cytokines, this inhibitor has an anti-inflammatory effect that could restrain the explosive state of inflammatory markers after infection [112, 113]. To increase TIMP-3 bioavailability, different approaches were conducted, like injecting recombinant TIMP-3 or through a mediated adenovirus gene [114, 115]. Therefore, TIMP-3 wide substrates and targets may imbalance some of the signaling pathways and mechanisms. These experiments are able to change radically the cell behavior by cleaving and shedding cell surface proteins including receptors, disintegrins and chemotactic elements. That is why a determined interval

FOI 26-3132 - Document 5

of its circulating level could offer a good prognostic marker since higher level implies also an ECM disequilibrium with several harmful consequences [110]. COVID-19 infections could induce an alveolar damage and enhance vascular permeability presumed in progressive hypoxemia, an ARDS or even an acute lung injury. As a consequence of immune cells activation and overexpression of cytokines, proteases, a probable acute lung injury (ALI) is established [116, 117]. ALI is characterized by a severe damage of alveolar barrier and mainly caused by an enhanced inflammatory response. Ventilated associated lung injury (VALI) has shown to be a common complication for COVID-19 patients and this can be only for worse [117]. To demonstrate MMPs implication in ALI and their probable potential effect in therapeutic experiments, animal models were established on clinical perturbations. On a deficient MMP-8 mice model and by treatment with an MMP-8 inhibitor, there was an improvement in gas exchange and a reduced damage after induced-ventilator lung injury [118]. However, Dolinay and his collaborators found that mice with an MMP-8 deficiency developed a higher alveolar permeability after the mechanical ventilation than wild type mice [118]. In the same context, an induced lung injury by immune cells infiltration in MMP-9-deficient mice model showed a lower state of lung injury than wild type [119, 120]. In fact, this gelatinase is probably implicated in decreasing pulmonary edema through healing the alveolar epithelium [121]. On the other hand, in response to a severe injury in lungs, fibrocytes and macrophages enhance collagen availability. The MMP-3 is considered to be the pro-generator of other MMPs since it is responsible for their activation. This stromelysin-1 is implicated in reducing lung injury level in MMP-3-deficient mice through the reduction of the chemotactic activity. Chemotactic peptides generated by neutrophils were upregulated in MMP-3-deficient mice including macrophage inflammatory protein and the complement chemotactic (C5a) [122]. Furthermore, there has been evidence that MMP-12 known also as the macrophage elastase is able to regulate monocytes influx in lungs through generating elastin fragments that play the role of chemotactic elements for immune cells recruitment in a chronic lung injury and smoking model [122].

SARS-CoV-2 new variant and matrix remodeling?

Even though the emerging new variant of SARS-CoV-2, the SARS-CoV-2 VUI 20201201 (Variant Under Investigation, year 2020, month 12, variant 01) [123], is yet under investigation and by the limited existent data, we thought of elaborating this actuality from another perspective. This new variant has been identified via viral genomic sequencing and is characterized by several spike proteins mutation, namely deletion 144, deletion 69–70, N501Y, A570D, D614G, T716I, P681H, D1118H, S982A [124]. Preliminary investigations suggest that its probable increased transmissibility could reach the 70%. Among these mutations, the N501Y is capable of increasing the ACE2 affinity since it touches the contact residues of the receptor-binding domain and seems to be highly related to virulence and infectivity factors in mice models [125, 126]. As for the P681H mutation, it creates a new furin cleavage site among the S1 and S2 and this site appears to be absent in other coronaviruses. This genetic variation is probably capable of promoting the viral entry in epithelial respiratory cells [17, 127, 128]. As for the 69–70 spike deletion, it has been shown to be associated to multiple RBD mutations [128, 129]. In summary, these mutations do enhance viral infiltration in host cells which explains its high transmissibility but do not alter the infection severity until the recent findings [130]. Based on these limited data, we can suggest that matrix remodeling after SARS-CoV-2 and SARS-CoV-2 VUI 20201201 will take the same road. Both of them will engender matrix destruction in a first way after activating the first line of defense which is presumed in an innate response. Afterwards, the real worry could be in activating more matrix mediators that will probably increase their secretion in a sudden way and set off several complications. It remains for us to investigate their evolution in the periods to come since there are still not enough data for this cluster.

Conclusion

Matrix remodeling has been widely implicated in several physiological, pathological and homeostasis state. Its mediators are capable of changing the fate of an organ since they are present and implicated in cell–cell and in a circulating way. Coronavirus invasion in the human body did alter matrix equilibrium. After elucidating matrix remodeling from this point of view, it is clearer that these mechanisms are pathologically modified during SARS-CoV-2 emerging. Since the lack of data, it will be interesting to focus on MMPs and their inhibitors for a therapeutic journey and to consider them as probable targets for a long-term treatment as cured patients remain with sequels.

Acknowledgements Illustrated figures were technically performed by Nesrine Fourti, a Phd student in our Biochemistry Department and Research Laboratory in la Rabta Hospital University in Tunis. We also thank Pr Alexandre Mebazaa from the INSERM UMRS 942, Department of Anesthesia and Critical Care in the Lariboisière Hospital, APHP, Paris, France (University Paris Diderot, Sorbonne Paris), France and Dr Charles de Roquetaillade from the Reanimation department in

the Andre Mignot Hospital in France for their effort in revising and correcting the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest and no financial support regarding this review project.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Safety, Tolerability, and Pharmacokinetics of Escalating High Doses of Ivermectin in Healthy Adult Subjects

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*Safety and pharmacokinetics (PK) of the antiparasitic drug ivermectin, administered in higher and/or more frequent doses than currently approved for human use, were evaluated in a double-blind, placebo-controlled, dose escalation study. Subjects (*n = *68) were assigned to one of four panels (3:1, ivermectin/placebo): 30 or 60 mg (three times a week) or 90 or 120 mg (single dose). The 30 mg panel (range: 347-594* μg/kg*) also received a single dose with food after a 1-week washout. Safety assessments addressed both known ivermectin CNS effects and general toxicity. The primary safety endpoint was mydriasis, accurately quantitated by pupillometry. Ivermectin was generally well tolerated, with no indication of associated CNS toxicity for doses up to 10 times the highest FDA-approved dose of 200* μg/kg*. All dose regimens had a mydriatic effect similar to placebo. Adverse experiences were similar between ivermectin and placebo and did not increase with dose. Following single doses of 30 to 120 mg, AUC and* C_{max} *were generally dose proportional, with* t_{max} *~4 hours and* $t_{1/2}$ *~18 hours. The geometric mean AUC of 30 mg ivermectin was 2.6 times higher when administered with food. Geometric mean AUC ratios (day 7/day 1) were 1.24 and 1.40 for the 30 and 60 mg doses, respectively, indicating that the accumulation of ivermectin given every fourth day is minimal. This study demonstrated that ivermectin is generally well tolerated at these higher doses and more frequent regimens.*

Journal of Clinical Pharmacology, 2002;42:1122-1133
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Ivermectin is a derivative of the avermectins, a family of macrocyclic lactones produced by the filamentous bacterium *Streptomyces avermitilis*. It is a highly effective antihelmintic agent used in animals and humans. Ivermectin was first approved for human use in 1987 and is donated by Merck & Co., Inc., as Mectizan® for the treatment of onchocerciasis and, more recently, lymphatic filariasis in Africa and Latin America. More than 200 million treatments have been distributed through the program. It is also approved as Stromectol® for the treatment of strongyloidiasis of the intestinal tract and onchocerciasis. Recently, approved human indications have been extended in France to include the treatment of scabies. The approved treatment regimens are as follows: 150 μg/kg once yearly for onchocerciasis, 200 μg/kg single dose for strongyloidiasis, and 150 to 200 μg/kg twice yearly or 300 to 400 μg/kg once yearly in endemic areas for lymphatic filariasis. The greatest experience with human use of ivermectin is in the dose ranges of 150 to 200 μg/kg.

Early pharmacokinetic studies conducted to support registration of the drug as a therapeutic agent against onchocerciasis demonstrated that plasma concentrations are approximately proportional to the dose, following oral administration of 6, 12, or 15 mg of ivermectin.[1] In two studies, after single 12 mg doses of ivermectin (2 × 6 mg) in fasting healthy volunteers (representing a mean dose of 165 μg/kg), the mean peak

From Merck Research Laboratories, Blue Bell and West Point, Pennsylvania, and Terlings Park, United Kingdom (Dr. Guzzo, Ms. Furtek, Dr. Porras, Dr. Chen, Mr. Tipping, Ms. Clineschmidt, Dr. Sciberras, Dr. Hsieh) and Clinical Pharmacology Associates, Miami, Florida (Dr. Lasseter). This study was sponsored by a grant from Merck Research Laboratories, a division of Merck & Co., Inc. Dr. Lasseter has no financial interest in Merck & Co. Dr. Sciberras was employed by Merck & Co., Inc., at the time of the study. All other authors are currently employed by Merck & Co., Inc. Submitted for publication March 5, 2002; revised version accepted June 30, 2002. Address for reprints: Ms. Christine Furtek, Merck & Co., Inc., BLX-29, P.O. Box 4, West Point, PA 19486.

DOI: 10.1177/009127002237994

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plasma concentrations of the major component (H_2B_{1a}) were 46.6 ± 21.9 (range: 16.4-101.1) ng/ml and 30.6 ±15.6 (range: 13.9-68.4) ng/ml, respectively, at approximately 4 hours after dosing.[2] Ivermectin is metabolized in the liver, and ivermectin and/or its metabolites are excreted almost exclusively in the feces over an estimated 12 days, with < 1% of the administered dose excreted in the urine. Estimates of plasma half-life have varied from about 12 to 56 hours.[3-7]

Oral ivermectin has been considered as a possible treatment for head lice. In recent years, this condition has become a significant problem in developed countries, resulting in school absenteeism, social distress, and stigmatization. The economic loss to parents can also be substantial; informal estimates from the Centers for Disease Control and Prevention show that the cost of lost work time due to head lice infestations exceeds $1 billion annually.[8] Initial exploratory studies evaluated ivermectin doses of 100 to 200 μg/kg.[9-11] However, none of these early studies demonstrated eradication of head lice, suggesting that higher or more frequent doses may be necessary for this use.

Since the majority of safety and pharmacokinetic data on oral ivermectin are with single doses of 150 to 200 μg/kg, the primary objective of this study was to obtain additional information at higher and multiple doses. Doses up to 10 times the 200 μg/kg dose were evaluated to provide a substantial safety margin over the anticipated dosage range for head lice treatment. Specifically, for assessment of safety, signs observed in animal toxicity studies, as well as in documented human overdose cases, were chosen as safety endpoints. In particular, mydriasis was evaluated as the primary safety endpoint because it could be accurately measured with a previously validated method, pupillometry. The pharmacokinetic objectives were to evaluate the pharmacokinetics of ivermectin with higher doses, the extent of any ivermectin accumulated with multiple doses, and the effect of food and gender.

METHODS

Subjects

Both male and female subjects were considered eligible to participate if they were between 18 and 45 years of age and in good health on the basis of history, physical examination, and routine laboratory data. Female subjects of childbearing potential agreed to use an effective double-barrier birth control method from at least 1 month prior to the start of the study until 1 month after the completion of the study. Weight requirement for subjects varied with treatment panel: 50 to 90 kg for panels 1 and 2 and 60 to 90 kg for panels 3 and 4. All subjects must have been nonsmokers for ≥ 6 months.

Subjects were ineligible for enrollment if they had any ocular abnormality that might interfere with pupillometry assessment, including corrected visual acuity worse than 20/30 in both eyes (contact lenses acceptable), a current eye disorder requiring the attention of an ophthalmologist, or a history of intraocular surgery. Pregnant or breast-feeding women were excluded. Subjects with a history of neurologic or CNS disease; treated or untreated hypertension, asthma, or other pulmonary disease; major gastrointestinal abnormalities/peptic ulceration; cardiovascular, hepatic, or renal disease; drug allergy; drug or alcohol abuse; or any illness that, in the opinion of the investigator, might have confounded the results of the study or posed additional risk in administering ivermectin to the subject could not participate. Other exclusion criteria included donation of a unit of blood or participation in another clinical trial with an investigational agent within 4 weeks prior to commencement of the study, regular use of any illicit drugs, intake of excessive amounts of caffeinated coffee or beverages (> 8 cups/day), or use of prescription or nonprescription medicine that could not be discontinued 2 weeks before the start of the study. Subjects with a known hypersensitivity to any component of the ivermectin product or who had any condition that the investigator felt might interfere with participation were not eligible.

This study was conducted at Clinical Pharmacology Associates (Miami, FL) in conformance with applicable country or local requirements regarding ethical committee review, informed consent, and other statutes or regulations regarding the protection of the rights and welfare of human subjects participating in biomedical research. The study protocol and subject consent form were approved by a private, nonaffiliated institutional review committee (Southern Institutional Review Board, Inc., Miami, FL), which was properly constituted according to the applicable regulations. Written informed consent was obtained from each subject.

Study Design

This was a double-blind, placebo-controlled, multiple rising dose study in healthy adult men and women. Subjects were sequentially assigned to one of four treatment panels and were randomized to receive ivermectin or placebo within each panel (12 active, 4 placebo per panel) using a computer-generated allocation schedule. Subjects were also stratified by gender within each panel to ensure that equal numbers of sub-

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jects of each gender were assigned to each treatment regimen.

Ivermectin was administered as a fixed dose extending from 30 mg in panel 1 to 120 mg in panel 4. Doses consisted of ten to forty 3 mg tablets of ivermectin as necessary to achieve the stipulated dose. When considering the weight limitations of the protocol, this corresponded to the potential delivery of 333 μg/kg to 2000 μg/kg. Actual dose range delivered (μg/kg) in enrolled subjects in each dose group is indicated in the following descriptions of the dosing regimens. Panel 1 subjects received treatment A: ivermectin 30 mg (347-594 μg/kg) or placebo, three times a week (days 1, 4, 7), in the fasted state during study week 1. After a washout period of at least 1 week, they then received treatment E: ivermectin 30 mg (347-541 μg/kg) or placebo, single dose, in the fed state during study week 3. Panel 2 received treatment B: ivermectin 60 mg (713-1091 μg/kg) or placebo, three times a week (days 1, 4, 7), in the fasted state during study week 5. Panel 3 received treatment C: ivermectin 90 mg (1031-1466 μg/kg) or placebo, single dose, in the fasted state during study week 9. Panel 4 received treatment D: ivermectin 120 mg (1404-2000 μg/kg) or placebo, single dose, in the fasted state during study week 11.

The treatment regimens were selected based on the possibility that doses higher than 200 μg/kg would be needed to eradicate head lice. The 30 and 60 mg doses were administered to evaluate safety in potential therapeutic dose ranges for head lice treatment, spanning 347 to 1091 μg/kg for the participants in this study. The regimen for these dose levels was the maximum frequency anticipated for head lice treatment (three doses in 7 days) and allowed for assessment of possible accumulation. The 90 and 120 mg doses were evaluated only to assess toxicity and provide an additional margin of safety; therefore, these treatments were administered as single doses.

Confirmation of quantifiable pupillometry was not initially an entrance criterion for the study. However, after completion of the original 16 subjects in panel 1, it was observed that pupillometry measurements for 5 subjects were difficult to quantify due to the unexpected extreme pigmentation of the iris in this particular study population. For one of these subjects, accurate pupil measurements could not be obtained. Therefore, the protocol was amended to add an entry criterion specifying that subjects must have a quantifiable pupil measurement at the prestudy visit (1-2 weeks prior to study start). Pupillometry was added to the prestudy visit procedures to accommodate this requirement. In addition, since testing of the primary hypothesis for this study was dependent on the pupillometry measurements from this subject panel, the protocol amendment also enrolled 4 additional subjects to panel 1 to compensate for the missing data. These 4 subjects received treatment A only (30 mg or placebo, days 1, 4, 7, fasted) and were dosed concurrently with panel 2. They were not administered treatment E (30 mg or placebo, single dose, fed) because the primary hypothesis related to treatment A only. A group of 4 subjects was considered appropriate because a blocking factor of 4 was used for the allocation schedule (3 active: 1 placebo). In addition, at the time of the amendment, it was unknown if more than 1 subject would have missing data.

All treatment doses were administered in the Clinical Research Unit (CRU) under medical supervision. Before each treatment period, subjects reported to the CRU at a predetermined time the evening prior to dosing on day 1. Subjects were required to fast from all food and liquid, except water, from midnight before all treatment days. Study drug was provided as ivermectin 3 mg tablets and matching placebo tablets and was administered with a total of 16 ounces of water to accommodate the large numbers of tablets administered at the higher doses. For consistency, the same amount of water was administered for all dose levels. For treatment E, subjects consumed a standard high-fat breakfast (31.3 g protein, 57.16 g carbohydrates, 48.6 g fat, 784 kcal) beginning 20 minutes before dosing. The dose was administered within 5 minutes after completion of the meal. For all other treatments, fasting (except for water) continued on all treatment days until the 4-hour plasma sample was collected, after which subjects consumed a meal of approximately 500 calories. Subjects resumed regular meals after the 8-hour plasma sample had been collected. Standardized meals were provided in the CRU at scheduled times.

Safety monitoring included careful clinical observation for adverse experiences as well as physical examinations (1-2 weeks prestudy [PS] and 5-7 days posttreatment [PT]), neurological examinations (PS; –1, 4, and 12 h; and PT), pupillometry (PS; –1, 4, 6, and 24 h; and PT), vital signs (PS; –1, 0, 1, 2, 4, 8, and 24 h; and PT), electrocardiograms (PS, –1 and 4 h, and PT), and clinical laboratory tests consisting of a CBC, blood chemistry, and urinalysis (PS, –1 h, and PT). In the neurological exam, tests to detect signs of ivermectin toxicity were emphasized and accurately quantitated when possible (e.g., heel-toe to evaluate ataxia). Details of pupillometry are described in the next section. All adverse events were recorded. The investigator paid par-

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ticular attention to any evidence of CNS toxicity, such as vomiting, mydriasis, or gait disturbance.

Treatment panels were dosed sequentially so that safety was established at a lower dose level (given in the fasted state) before proceeding to the next higher dose level. Safety parameters were chosen based on experience with human overdose and animal toxicity. For an individual subject, the investigator was instructed to discontinue a subject's test drug if any of the following signs or symptoms were observed postdose: one or more episodes of drug-related vomiting (excluding episodes occurring within 1 h of dosing), mydriasis (defined as absence of change in pupil size upon transition from low-light to high-light conditions during pupillometry), or gait disturbance (defined as a confirmed abnormal result from heel-toe test as compared with baseline prestudy assessment). For a dosage panel, the investigator was instructed to discontinue advancement to the next dose level if any of the following situations occurred: mydriasis or gait disturbance (as defined above for an individual subject) was observed in 1 or more subjects, a ≥ 2 mm increase in pupil diameter over baseline (predose) was observed in 2 or more subjects, or drug-related vomiting (excluding episodes occurring within 1 h of dosing) was observed in 3 or more subjects in a panel. In addition, the incidence of one or more drug-related serious adverse experiences or two or more drug-related severe adverse experiences in a panel would have warranted consideration of discontinuing dose-level advancement.

For pharmacokinetic analysis, plasma samples were collected at predefined intervals on days 1, 4, and 7 for treatments A and B and on day 1 only for treatments C, D, and E and analyzed for ivermectin concentration. The predefined intervals were predose (0 h) and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48, and 60 hours postdose on days 1 and 7 and predose only on day 4.

For treatments A and B, subjects remained in the CRU until the final day 7 blood sample was obtained (60 h after the day 7 dose). For treatments C, D, and E, subjects remained in the CRU until the final day 1 blood sample was obtained (60 h after the day 1 dose). Subjects returned for a posttreatment assessment 5 to 7 days following the last dose of study drug in a treatment period.

Pupillometry

To monitor for any evidence of mydriasis, this study used a method of pupillometry that had been validated internally and successfully used in a previous study of a mydriatic agent. The method accurately and reproducibly measures pupil diameter by photograph under standard lighting conditions. Photographic equipment consisted of a single-lens reflex camera (Nikon F70 camera body and Nikon 105 micro lens) with flash unit (Nikon SB23 with remote link), mounted on a support device in front of and facing the subject with the camera lens projecting through a reflective lighting screen. The subject's head was positioned on a head rest (similar to those found in a slit lamp unit). To reduce the influence of accommodation movement on the pupil, subjects focused through a hole in the reflective screen on an object 3 m beyond the camera.

All pupil measurements were made in a room equipped with black-out shades. Standard lighting conditions were created using two banks of four low-voltage tungsten lamps, projected from behind the subject onto a white screen located approximately 0.5 m in front of the subject. Dimmer switches attached to each bank of lights enabled the lamp units to be adjusted to either low-light (10-foot candles) or high-light (200-foot candles) conditions. A calibrated light meter (Gossen) placed at the temple of the subject's head, adjacent to the eye, was used to confirm that the proper light levels reached the subject's corneal plane. The left eye was photographed under both standard lighting conditions, in duplicate, at each time point prescribed by the protocol. In cases where a subject's corrected visual acuity was worse than 20/30 in the left eye, the right eye was photographed. Measurements of the maximal horizontal diameter of the pupillary area were obtained via image analysis of the developed transparencies (MCID M1 image analysis system, Imaging Research Inc., Ontario) and recorded in millimeters for statistical analysis. Photographs taken of a measuring scale (graph paper), placed at the same eye-to-camera distance used for pupil measurement, were used for calibration.

Prior to enrolling subjects into this study, pupil measurement standards and reproducibility were established at the study site. The method was tested under the same lighting conditions and in the same examination room subsequently used in performing the pupil measurements for the clinical study. Prestudy testing of the photographic measurement methodology was conducted by the same study personnel who performed pupil measurements during the study.

In addition, visual accommodation was measured predose for all subjects to obtain a baseline value (before the first dose of study drug only). Subsequently, if mydriasis was observed in a subject at any time during the study, accommodation was to be measured (as soon as possible after observation of mydriasis) to determine if there was accompanying cycloplegia.

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FOI 26-3132 - Document 5

Ivermectin Assay

Plasma was separated by centrifugation from blood samples collected from the original 48 subjects on active treatment, at the time points specified above, and analyzed for ivermectin H_2B_{1a}. The additional 4 subjects added per protocol amendment were for safety assessment only, and samples from these subjects were to be assayed only in the event of a drug-related or serious adverse experience. Assay of plasma samples for ivermectin H_2B_{1a} concentration was performed by the Drug Metabolism Department, Merck Research Laboratories (West Point, PA), using a high-performance liquid chromatography (HPLC) method with fluorescence detection. The protein was removed from plasma samples by precipitation with acetonitrile. Ivermectin and the internal standard were then recovered from the matrix by liquid/solid-phase extraction on ChemElut cartridges. Reconstituted extracts were online derivatized and separated by reverse-phase HPLC and quantitated by fluorescence detection.

A standard curve of 0.5, 1, 2.5, 5, 10, 20, and 40 ng/ml ivermectin spiked into human control plasma was analyzed daily along with clinical samples. Accuracy ranged from 98.6% to 102% of nominal concentrations; precision ranged from 2.2% to 5.9% through the entire curve. Plasma quality control samples at low (1.25 ng/ml), medium (7.5 ng/ml), and high (25 ng/ml) concentrations were prepared prior to the start of the study and subjected to replicate within-day analyses (n = 5). Two high, two medium, and two low quality control samples were analyzed daily along with clinical samples to assess interday variability. Accuracy averaged 99.9%, 104.7%, and 102.6% for the low, medium, and high quality control samples, respectively. The corresponding values for precision were 7.9%, 6.1%, and 4.7%, respectively. The limit of reliable quantification for ivermectin H_2B_{1a} was 0.5 ng/ml.

Pharmacokinetic Calculations

The following pharmacokinetic parameters were evaluated: area under the plasma concentration-time curve from time 0 to infinity ($AUC_{0-\infty}$), maximum plasma concentration (C_{max}), time of occurrence for maximum (peak) plasma concentration (t_{max}), and half-life ($t_{½}$). $AUC_{0-\infty}$ was estimated by the trapezoidal method to the 60-hour point and extrapolated to infinity by the half-life estimated for that curve. C_{max} and t_{max} values were estimated by inspection of the plasma concentration-time curve. Half-life was estimated by the method of Kwan et al.[12] Accumulation ratios were approximated from ratios of $AUC_{0\text{-}60\ h}$ since these were the closest values available to the ratio of AUC_{τ} (τ = 72 h) for this study. Computed values of AUC and half-life were calculated in EXCEL (Microsoft Corporation, Redmond, WA).

Statistical Analysis

Safety

The primary parameter for the safety assessment of ivermectin was change in pupil size under high-light conditions between baseline (day 1 predose) and the approximate time of maximum drug concentration (C_{max}) on day 7 (maximum of the 4- and 6-h time points). The primary hypothesis was that oral administration of ivermectin in the fasted state, at a regimen of 30 mg for three doses over 1 week (days 1, 4, 7), does not affect the maximum pupil diameter change from baseline at high-light conditions on day 7 as compared to placebo; a mean difference of 1 mm between the 30 mg ivermectin and placebo groups was considered significant. The difference in maximum (of the 4- and 6-h time points) pupil diameter change from baseline (day 1 predose) at high-light conditions on day 7 between the groups receiving 30 mg ivermectin (in the fasted state) and placebo (pooled from treatments A and B) was assessed using an analysis of variance (ANOVA) model with gender, treatment, and gender-by-treatment interaction as factors. A 90% confidence interval for the mean difference (30 mg ivermectin [in the fasted state] vs. placebo) in maximum (of the 4- and 6-h postdose time points) pupil diameter change from baseline (day 1 predose) at high-light conditions on day 7 was calculated using the MSE from the ANOVA referencing a t-distribution. Whether or not to combine the results from males and females within each treatment was determined by assessing the gender-by-treatment interaction. The assumption of normality was tested using the Shapiro-Wilk statistic.

Given a parallel study with 12 subjects in one group (30 mg fasted) and 8 in the other (placebo) and similar between-subject variation (mean square error = 0.0361) as estimated from historical data, there is a > 99% probability that the observed 90% confidence interval for the mean difference in maximum pupil diameter change from baseline (day 1 predose) between the 30 mg ivermectin (in the fasted state) group and the placebo group would fall within the equivalence interval of (–1.0, 1.0), if the true difference was 0. Probabilities were calculated using SAS 6.12 (SAS Institute, Cary, NC).

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Although not specified in the primary hypothesis, an analysis of the pupil diameter changes for the other 30 mg time points (days 1 and 4, high light; days 1, 4, and 7, low light) and for the other dose groups was done using the same criteria as for the primary analysis.

General safety and tolerability were assessed by examining adverse experiences and laboratory data. Adverse experiences were tabulated and summarized.

Pharmacokinetics

Estimation of the pharmacokinetic parameters of ivermectin in this four-panel, escalating dose study was accomplished by using an ANOVA model with gender, dose, and gender-by-dose interaction as factors. The analysis of the day 1 data included data from treatments A, B, C, and D. The day 7 analysis included data from only treatments A and B. Whether or not to combine the results from males and females within each treatment was determined by assessing the gender-by-dose interaction. The assumption of normality was tested using the Shapiro-Wilk statistic.

The effect of food on the pharmacokinetics of ivermectin on day 1 was estimated by comparing the natural log-transformed $AUC_{0-\infty}$ values for the 30 mg dose of ivermectin in the fed and fasted states (treatment E vs. treatment A). An ANOVA model, including treatments A and E with subject and treatment as factors, was used. A 95% confidence interval for the AUC geometric mean ratio (30 mg ivermectin fed [treatment E]/30 mg ivermectin fasted [treatment A]) was calculated using the MSE from the ANOVA referencing a *t*-distribution.

Ivermectin accumulation was assessed in the following manner: an ANOVA model including treatments A and B with dose, day, and dose-by-day interaction as factors was used to analyze the natural log-transformed $AUC_{0\text{-}60\ h}$ values. Ninety-five percent confidence intervals for the AUC geometric mean ratio (day 7, last dose/day 1, first dose) were calculated using the MSE from the ANOVA referencing a *t*-distribution for the 30 mg ivermectin fasted (treatment A) and 60 mg ivermectin (treatment B) treatment groups.

RESULTS

Subjects

Sixty-eight subjects (51 ivermectin, 17 placebo) entered the study and were sequentially assigned to one of four treatment panels. The subjects' ages ranged from 21 to 45 years, with a median of 34.5 years. Thirty-five subjects (51.5%) were male and 33 subjects (48.5%) were female. Subjects were 89.7% Hispanic, 4.4% Caucasian, 4.4% Black, and 1.5% Asian. The distribution of age, gender, and racial origin appeared to be similar across the treatment groups. Overall, the study population was generally healthy with few medical problems. Secondary diagnoses were similar across the treatment groups, consisting primarily of a history of cesarean section or tubal ligation.

Safety

Pupillometry

Table I summarizes pupil diameter changes from baseline (day 1 predose) in all study groups postdose (days 1, 4, and 7, when measured) at both low-light and high-light conditions. The mean pupil diameter in the ivermectin 30 mg (fasted) treatment regimen decreased slightly from baseline for both males (–0.119 mm) and females (–0.107 mm). The mean pupil diameter in the placebo regimen remained virtually unchanged from baseline for both males (–0.002 mm) and females (0.035 mm). Gender effect was not significant at the 95% confidence level from an analysis of variance (ANOVA) model with gender, treatment, and gender-by-treatment interaction as factors.

The difference in pupil diameter change from baseline (day 1 predose) at high-light conditions on day 7 between the groups receiving 30 mg ivermectin in the fasted state and placebo (pooled from treatments A and B) was –0.013. The 90% confidence interval (–0.239, –0.021) for the difference falls within the equivalence interval (–1.0, 1.0), indicating that the ivermectin 30 mg regimen in the fasted state had an equivalent mydriatic effect as compared with placebo, according to prespecified criteria. Exploratory examination of pupil size of all other groups on days 1, 4, and 7 at both high-light and low-light conditions confirmed that there was no evidence of a mydriatic effect.

Adverse Events

Clinical adverse experiences reported during the study are presented in Table II. Of the 51 subjects who received ivermectin, 12 subjects (24%) reported at least one clinical adverse experience. This rate was similar to that observed in the placebo group (6 subjects, 35%). In addition, there was no consistent trend in the incidence of adverse experiences indicative of a dose response. All clinical adverse experiences were transient and mild, and no adverse experience recurred with repeated dosing. The most commonly reported adverse experiences were headache, nausea, dizziness, and rash, occurring in both ivermectin- and placebo-treated

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Table I Mean (*SD*) of Pupil Diameter Changes (mm) from Baseline by Treatment Regimen and Light Conditions

	Placebo[a]	Ivermectin 30 mg (fasted) (*n* = 15)	Ivermectin 60 mg (fasted) (*n* = 12)	Ivermectin 90 mg (fasted) (*n* = 12)	Ivermectin 120 mg (fasted) (*n* = 12)	Ivermectin 30 mg (fed) (*n* = 11[b])
At high-light conditions						
Day 1	0.064	–0.103	0.050	0.088	0.020	0.013
	(0.167)	(0.165)	(0.132)	(0.104)	(0.129)	(0.089)
Day 4	0.001	–0.021	–0.097	NA	NA	NA
	(0.146)	(0.263)	(0.136)			
Day 7	0.016	–0.114	–0.036	NA	NA	NA
	(0.155)	(0.140)	(0.294)			
		(–0.239, –0.021)[c]				
At low-light conditions						
Day 1	0.139	0.050	0.151	0.273	0.237	–0.009
	(0.471)	(0.382)	(0.363)	(0.417)	(0.207)	(0.283)
Day 4	0.113	–0.016	–0.028	NA	NA	NA
	(0.469)	(0.486)	(0.339)			
Day 7	0.199	–0.081	0.095	NA	NA	NA
	(0.903)	(0.458)	(0.570)			

a. Excluding 1 subject from panel 1 whose pupillometry measurements were not quantifiable. For day 1, pooled from treatments A, B, C, and D, $n = 16$; for days 4 and 7, pooled from treatments A and B, $n = 8$.

b. One subject discontinued prior to receiving fed treatment and did not contribute to the corresponding analysis.

c. The 90% confidence interval (CI) for the primary endpoint (difference in mean change between ivermectin 30 mg, fasted and placebo at high-light conditions on day 7). The 90% CI falls within the predetermined equivalence interval of (–1.0, 1.0) mm.

subjects. Seven ivermectin-treated subjects (14%) and 3 placebo-treated subjects (18%) had drug-related clinical adverse experiences. None of the subjects discontinued due to clinical adverse experiences. None of the clinical adverse experiences was serious.

Two subjects experienced laboratory adverse experiences, both in the 30 mg ivermectin group. A 37-year-old female developed increased alanine transaminase (ALT) to approximately 2.5 times normal on day 14 and elevated gamma-glutamyl transferase (GGT) to approximately 4 times normal on day 18. ALT returned to normal on day 23, but GGT remained mildly elevated (no baseline values obtained). Further hepatic evaluation revealed cholelithiasis (a gallbladder filled with stones). The investigator rated both laboratory adverse experiences as possibly related to study drug. The second subject, a 25-year-old male, developed hematuria that resolved after passing a kidney stone, and the event was evaluated as definitely not related to study drug.

Two subjects developed elevated liver function tests (LFTs) at least two times the upper limit of normal. The first subject was in the 30 mg ivermectin group and developed increased ALT and GGT levels, which were reported as laboratory adverse experiences (described above). The second subject, a 38-year-old male on placebo treatment, developed increased aspartate transaminase (AST) and ALT levels to approximately two times normal on day 7. The investigator considered these elevations to be not clinically significant.

Two subjects discontinued the study. One subject in the 30 mg ivermectin group discontinued due to a laboratory adverse experience (37-year-old female described above). One subject in the placebo group was lost to follow-up.

Pharmacokinetics

General

Pharmacokinetic results ($AUC_{0-\infty}$, C_{max}, t_{max}, and half-life) are shown in Table III. In addition, box plots of $AUC_{0-\infty}$ by ivermectin dose (day 1, fasted only) are displayed in Figure 1. For single doses of 30 to 120 mg administered in the fasted state, the AUC and C_{max} of ivermectin increase with increasing dose and appear generally dose proportional in this range. A high variability in absorption is apparent from examination of

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Table II Clinical Adverse Experiences by Body System

	Placebo (n = 17)		Ivermectin 30 mg (n = 15)		Ivermectin 60 mg (n = 12)		Ivermectin 90 mg (n = 12)		Ivermectin 120 mg (n = 12)	
	Number	%	Number	%	Number	%	Number	%	Number	%
Subjects with one or more adverse experiences	6	35.3	5	33.3	5	41.7	2	16.7	0	0.0
Subjects with no adverse experience	11	64.7	10	66.7	7	58.3	10	83.3	12	100
Body as a whole/site unspecified	0	0.0	2	13.3	1	8.3	0	0.0	0	0.0
Fever	0	0.0	1	6.7	0	0.0	0	0.0	0	0.0
Flu-like illness	0	0.0	0	0.0	1	8.3	0	0.0	0	0.0
Pain, abdominal	0	0.0	1	6.7	0	0.0	0	0.0	0	0.0
Digestive system	1	5.9	1	6.7	1	8.3	1	8.3	0	0.0
Dry mouth	0	0.0	0	0.0	0	0.0	1	8.3	0	0.0
Fecal abnormality	0	0.0	1	6.7	0	0.0	0	0.0	0	0.0
Nausea	1	5.9	0	0.0	1	8.3	0	0.0	0	0.0
Vomiting	0	0.0	0	0.0	1	8.3	0	0.0	0	0.0
Musculoskeletal system	1	5.9	2	13.3	0	0.0	0	0.0	0	0.0
Pain, back	0	0.0	1	6.7	0	0.0	0	0.0	0	0.0
Pain, leg	1	5.9	0	0.0	0	0.0	0	0.0	0	0.0
Stiffness	0	0.0	1	6.7	0	0.0	0	0.0	0	0.0
Nervous system and psychiatric disorder	3	17.6	1	6.7	4	33.3	1	8.3	0	0.0
Anxiety	0	0.0	0	0.0	1	8.3	0	0.0	0	0.0
Dizziness	1	5.9	0	0.0	1	8.3	0	0.0	0	0.0
Headache	2	11.8	1	6.7	3	25.0	1	8.3	0	0.0
Skin and skin appendage	1	5.9	0	0.0	1	8.3	0	0.0	0	0.0
Rash	1	5.9	0	0.0	1	8.3	0	0.0	0	0.0
Urogenital system	0	0.0	1	6.7	0	0.0	0	0.0	0	0.0
Urolithiasis	0	0.0	1	6.7	0	0.0	0	0.0	0	0.0

Although a subject may have had two or more adverse experiences, the subject is counted only once within a category. The same subject may appear in different categories. All body systems are listed in which at least 1 subject had an adverse experience.

standard deviations and from the overall trends in AUC and C_{max} with increasing dose. Specifically, AUC and C_{max} do not appear to increase between the doses of 60 and 90 mg. However, a nearly proportional increase is seen with the 120 mg dose, indicating that absorption is not saturated in this range. t_{max} and half-life were generally invariant across the dose range, as would be expected from linear kinetics. There was no significant trend indicative of a gender effect.

Accumulation

The mean plasma concentration profiles on days 1 and 7 following multiple doses of 30 and 60 mg ivermectin given in the fasted state are shown in Figure 2. The geometric mean ratio (GMR) of the $AUC_{0\text{-}60\ h}$ of ivermectin on day 7 to the $AUC_{0\text{-}60\ h}$ on day 1 was calculated for assessing accumulation. GMRs (day 7/day 1) were 1.24 and 1.40 for the ivermectin 30 and 60 mg doses, respectively. This result suggests minimal accumulation of drug with day 1, 4, and 7 dosing, consistent with a short half-life relative to the dosing interval (72 h). The 95% confidence interval was (0.80, 1.92) and (0.91, 2.18) for the 30 mg (in the fasted state) level and the 60 mg level, respectively.

Food Effect

The effect of food on the pharmacokinetics of ivermectin was estimated by comparing the mean natural log-transformed $AUC_{0\text{-}\infty}$ values for the 30 mg dose of ivermectin in the fed and fasted states (treatment E vs. A). The mean plasma concentration profiles in the fed and fasted states are presented in Figure 3. The geo-

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Table III Mean (*SD*) of Pharmacokinetic Parameters by Treatment Regimen

Parameter	Ivermectin 30 mg (fed) (n = 11[b])	Ivermectin 30 mg (fasted) (n = 12)[a]		Ivermectin 60 mg (n = 12)		Ivermectin 90 mg (n = 12)	Ivermectin 120 mg (n = 12)
		Day 1	Day 7	Day 1	Day 7		
$AUC_{0-\infty}$ (ng•h/ml)[c]	4564.6	1724.3	2819.4	2984.0	6061.7	2910.2	4547.7
	(1892.5)	(830.5)	(1691.2)	(1530.1)	(4243.7)	(1801.9)	(2402.9)
AUC_{0-60} (ng•h/ml)[d]	NA	1166.3	1444.3	2099.3	2947.2	NA	NA
$AUC_{0-\infty}$ (ng•h/ml)[d]	3951.9	1538.4	NA	NA	NA	NA	NA
C_{max} (ng/ml)[c]	260.5	84.8	87.0	165.2	186.2	158.1	247.8
	(172.1)	(42.7)	(43.2)	(98.6)	(130.8)	(87.6)	(158.9)
$t_{1/2}$ (h)[e]	15.0	20.1	17.7	19.6	17.5	18.8	19.1
t_{max} (h)[c]	4.6	4.3	4.2	3.6	4.0	4.9	4.2
	(0.9)	(1.0)	(0.9)	(0.9)	(1.1)	(1.8)	(0.9)

a. As prespecified in the protocol amendment, the plasma samples from the 3 additional subjects receiving 30 mg ivermectin treatment were not analyzed and did not contribute to the pharmacokinetic analyses.
b. One subject discontinued prior to receiving fed treatment and did not contribute to the corresponding analysis.
c. Arithmetic mean (standard deviation).
d. Geometric mean.
e. Harmonic mean.

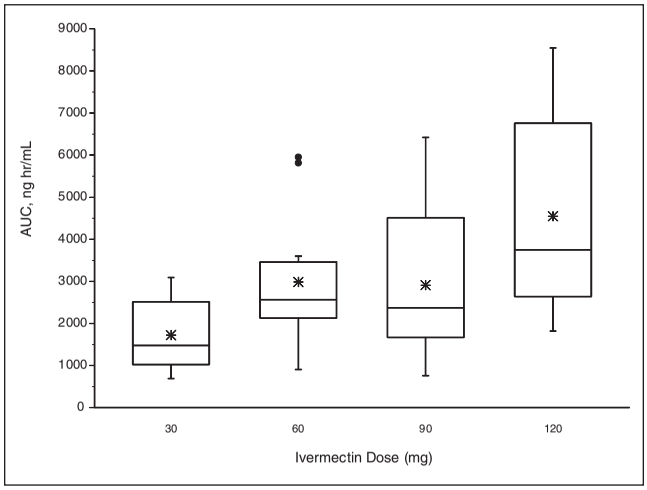


Figure 1. Box plots of AUC by ivermectin dose (day 1, fasted only). The box represents the middle half of the data with the bottom and top of the box representing the 25th and 75th percentiles. The median is portrayed by a horizontal line segment within the box, and the mean is represented by an asterisk. The height of the box is multiplied by 1.5 to determine the height of the "step." The upper vertical line extends up to the highest data value within the step, and the lower vertical line extends down to the lowest data value within the step. Circles represent data values that exceed the 25th and 75th percentiles by more than the height of the step. Mean AUC does not appear to increase between the doses of 60 and 90 mg, largely due to the influence of the two outliers in the 60 mg dose group. A nearly proportional increase is seen with the 120 mg dose, indicating that absorption is not saturated in this range.

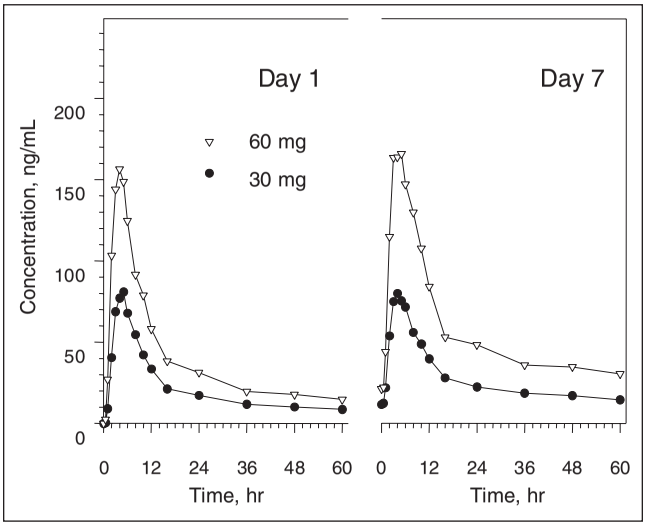


Figure 2. Mean plasma concentration (ng/ml) profiles of ivermectin on days 1 and 7 following multiple oral doses of 30 and 60 mg given fasted on days 1, 4, and 7.

metric mean ratio of $AUC_{0-\infty}$ values (fed/fasted) was 2.57. The 95% confidence interval was (2.16, 3.05).

DISCUSSION

This study was designed primarily to evaluate the safety and tolerability of oral ivermectin to support its

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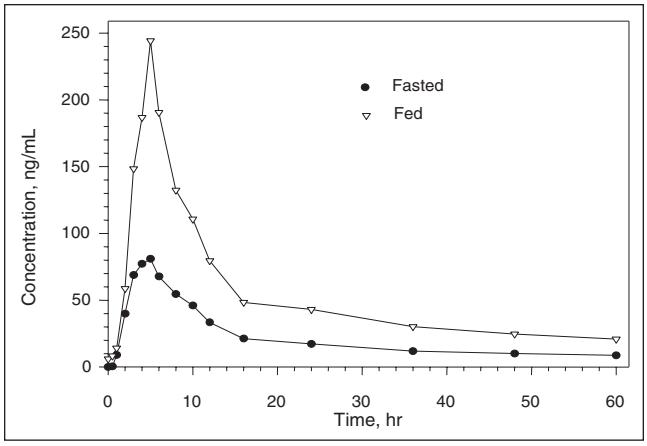


Figure 3. Mean plasma concentration (ng/ml) profiles of ivermectin following single oral doses of 30 mg (fed and fasted administration).

use for the treatment of head lice infestation. Head lice predominantly affect school-age children. However, for both ethical and practical reasons, it was felt appropriate to conduct this study in healthy adult subjects. Extensive safety data are available for ivermectin, primarily at dose levels of 150 to 200 µg/kg. There also are published studies conducted at doses higher than 200 µg/kg for the treatment of internal parasites.[13-21] The present study expanded the safety data available for ivermectin at higher doses in subjects without internal parasitic infestations. The safety and tolerability of ivermectin were evaluated from two perspectives in this study: (1) assessment of CNS effect, the sole target of previously documented ivermectin toxicity, and (2) assessment of the general toxicity of ivermectin. No CNS toxicity was detected in the study, and ivermectin was generally well tolerated. Therefore, this study demonstrates that ivermectin is well tolerated in healthy subjects and provides additional evidence that the more severe reactions observed in the treatment of onchocerciasis and lymphatic filariasis are most likely due to the death of the organisms.[22]

A second purpose of the study was to collect additional pharmacokinetic data on ivermectin. Specifically, the study was designed to extend the kinetic understanding of this drug beyond the doses (up to 15 mg) examined previously, to document its behavior when administered in repeated doses in the manner contemplated for use against head lice, and to examine the effect of a high-fat meal on absorption.

Safety and Tolerability

Specific signs or symptoms of CNS toxicity were based on those determined in previous high-dose animal studies and observations of human overdose and included emesis, mydriasis, and ataxia. Mydriasis was chosen as the endpoint for the primary hypothesis since it can be accurately quantitated through pupillometry. Pupillometry is a simple, accurate, and reproducible method to measure pupil diameter quantitatively, which was validated internally prior to use in this study. It was observed in the first treatment panel that measurement of pupil diameter was difficult in persons with extremely dark iris color. However, with the addition of a screening evaluation at the prestudy visit, this difficulty was resolved.

No indication of CNS toxicity associated with oral ivermectin was observed for any of the doses administered in this study. This was most strongly supported by the absence of a mydriatic effect using quantitative pupillometry. The primary analysis of the pupillometry data was in the 30 mg ivermectin treatment group since this fixed dose spanned the range (in µg/kg) most likely to be employed in head lice therapy. Comparison of pupil size to baseline was made after the third dose, when maximum drug concentration was likely to be present if any accumulation occurred. Considering this criterion, the change in pupil size following 30 mg ivermectin administration was not different from that observed with placebo. Escalation to a single dose of up to 2 mg/kg, 10 times the approved dose, was also unassociated with any mydriatic effect. This supports the safety of ivermectin in the anticipated dosage range for head lice and provides a significant margin of safety.

Ivermectin was generally well tolerated. There were no serious clinical or laboratory adverse experiences. Clinical adverse experiences were similar between the ivermectin and placebo groups.

Specific adverse experiences to consider are those within the gastrointestinal and nervous systems since emesis, ataxia, and mydriasis are cardinal signs of ivermectin toxicity. Three of 51 subjects in the ivermectin-treated groups (1 fecal abnormality, 1 nausea, 1 vomiting) and 1 of 17 subjects in the placebo groups (1 nausea) experienced gastrointestinal adverse experiences. The adverse experience of vomiting occurred in a subject administered 60 mg of ivermectin on day 4. The event was evaluated as not related to ivermectin and did not occur after the day 1 dose, nor

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did it recur with the day 7 dose. Six of the 51 subjects in the ivermectin-treated groups (4 headache, 1 anxiety, 1 dizziness) and 3 of the 17 subjects in the placebo groups (2 headache, 1 dizziness and headache) experienced nervous system adverse experiences. No increase in any adverse experiences was noted with dose escalation, and no adverse experience recurred with repeated dosing. Of note, no adverse experiences were reported in the subjects who received 120 mg of ivermectin.

Two subjects developed increases in LFTs during the study, 1 on ivermectin 30 mg and 1 on placebo. The subject on active drug was subsequently identified as having cholelithiasis, which was unlikely to be related to acute treatment with ivermectin. No other clinically significant LFT abnormalities occurred despite dose escalation to 120 mg. These data, together with extensive animal and human exposure, suggest that routine monitoring of laboratory values is unnecessary at the dose levels anticipated for head lice treatment.

Pharmacokinetics

Pharmacokinetic parameters of ivermectin following single doses were consistent with previous observations[1] and with linear behavior up to single doses of 120 mg. Substantial variability in absorption was apparent across doses, but this phenomenon was attributable to absorption rates of < 50% in the fasted state and to the parallel panel design of the study.

Absorption of ivermectin was significantly higher when administered following a high-fat meal, approximately 2.5 times that in the fasted state. Although the effect of food on absorption of ivermectin had not been previously studied, this result was not unexpected. When the drug was administered in a hydroalcoholic solution,[1] a similar increase in bioavailability was observed (compared to administration as a tablet). The extent of agreement between these results suggests that the poor solubility in water of the lipophilic ivermectin molecule limits absorption, but agents that help to solubilize the molecule (such as alcohol or bile) increase absorption of the drug. However, the standard high-fat meal is not representative of typical food ingestion. Furthermore, it is reassuring that despite a substantial increase in the AUC of ivermectin with administration of a high-fat meal, no toxicity was noted.

The apparent half-life for ivermectin identified in the product label is 16 hours or longer. The overall estimate from this study (approximately 18 h) is slightly longer. Half-life estimates varied somewhat with dose and means of administration from approximately 15 hours in fed subjects given 30 mg ivermectin doses to approximately 20 hours in the same group following administration of 30 mg ivermectin in the fasted state. Since the bioavailability is substantially higher when the drug is administered in the fed state, and a shorter half-life was observed in this state, it is possible that the half-life observed following oral administration is influenced by slow absorption of the drug, rather than being a true estimate of elimination half-life.

The accumulation observed following administration of drug every fourth day was rather modest and consistent with a moderate elimination half-life. The apparent change observed between the 30 and 60 mg dose regimens is likely attributable to the significant variability in absorption and the small number of subjects studied. In fact, accumulation ratios for both doses were statistically indistinguishable from 1. No significant pharmacokinetic differences were observed between men and women. No information has been gathered on the pharmacokinetics of ivermectin in children, but given the generally consistent kinetic behavior among many different species of widely distributed body sizes, it is expected that the pharmacokinetics in children will be consistent with those in adults.

In summary, the safety profile generated in this study supports the use of oral ivermectin at the approved dose levels and at dose levels being considered for the treatment of head lice. Furthermore, a significant safety margin is demonstrated. However, studies in the pediatric population are necessary to confirm safety in children at higher dose levels. The pharmacokinetic parameters are consistent with those previously established, with the exception of a slightly longer half-life. A significant food interaction was demonstrated.

We would like to acknowledge the following individuals for their considerable contributions to this study: Mr. Manuel Pinto and the staff of Clinical Pharmacology Associates for their invaluable help with study conduct and patient recruitment, Ms. Geraldine Bolden of Merck Research Laboratories for her excellent work in conducting the image analysis of the pupillometry transparencies, Ms. Mitra Asgary of Merck Research Laboratories for her contribution in analyzing the pharmacokinetic samples, and Dr. Stephanie Larouche, formerly of Merck Research Laboratories, for her encouragement and scientific guidance.

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Received: 16 May 2021 | Revised: 1 June 2021 | Accepted: 2 June 2021

DOI: 10.1002/rmv.2265

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REVIEW

Ivermectin and outcomes from Covid-19 pneumonia: A systematic review and meta-analysis of randomized clinical trial studies

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Summary

Ivermectin is an FDA-approved drug for a parasitic disease that has broad antiviral activity. This study aims to analyse the efficacy of ivermectin in improving the Covid-19 outcomes. We systematically searched the PubMed, Europe PMC and ClinicalTrials.gov database using specific keywords related to our aims until 10th May 2021. All published randomized clinical trial studies on Covid-19 and ivermectin were retrieved. The quality of the study was assessed using Jadad scale assessment tool for clinical trial studies. Statistical analysis was done using Review Manager 5.4 software. A total of 19 studies with 2768 Covid-19 patients were included in this meta-analysis. This meta-analysis showed that ivermectin was associated with reduction in severity of Covid-19 (RR 0.43 [95% CI 0.23–0.81], $p = 0.008$), reduction of mortality (RR 0.31 [95% CI 0.15–0.62], $p = 0.001$), higher negative RT-PCR test results rate (RR 1.23 [95% CI 1.01–1.51], $p = 0.04$), shorter time to negative RT-PCR test results (mean difference [MD] −3.29 [95% CI −5.69, −0.89], $p = 0.007$), higher symptoms alleviations rate (RR 1.23 [95% CI 1.03–1.46], $p = 0.02$), shorter time to symptoms alleviations (MD −0.68 [95% CI −1.07, −0.29], $p = 0.0007$) and shorter time to hospital discharge (MD −2.66 [95% CI −4.49, −0.82], $p = 0.004$). Our study suggests that ivermectin may offer beneficial effects towards Covid-19 outcomes. More randomized clinical trial studies are still needed to confirm the results of our study.

KEYWORDS
covid-19, ivermectin, treatment

Abbreviations: CI, confidence intervals; Covid-19, coronavirus disease 2019; FDA, The United States Food and Drug Administration; FiO2, fractional concentration of oxygen inspired air; ICU, intensive care unit; IgE, immunoglobulin E; IgG1, immunoglobulin G1; IL-1, interleukin 1; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; LPS, lipopolysaccharide; NCP, nucleocapsid protein; NF-kB, nuclear factor-kappa B; PaO2, partial pressure of arterial oxygen; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RR, risk ratio; RT-PCR, reverse transcriptase-polymerase chain reaction; SARS, severe acute respiratory syndrome; SARS-CoV-2, SARS-coronavirus-2; SD, standard deviations; TNF-α, tumour necrosis factor-α.

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1 | INTRODUCTION

At the end of 2019, the first cases of a newly discovered acute respiratory illness, named coronavirus disease 2019 (Covid-19), were reported in Wuhan, China. As of 22 December 2020, a total of about 75.1 million cases and 1,680,794 deaths were identified across the world.[1] Covid-19 has various clinical manifestations, ranging from mild respiratory manifestations such as fever, cough, anosmia to severe or life-threatening conditions such as shock, respiratory distress, arrhythmia, sepsis, loss of consciousness.[2–5] Previous published meta-analysis studies have identified several comorbidities,[6–10] home medications[11,12] and laboratory values[13,14] which are associated with severe outcomes and the risk of dying from Covid-19. To reduce the severity and mortality rate of Covid-19, many attempts have been undertaken, including to discover the potential therapy. There were many therapeutic agents evaluated in clinical trials and suggested for Covid-19 therapy, such as remdesivir, dexamethasone, colchicine and tocilizumab.[15–17] These drugs may be beneficial for Covid-19 treatment because of their effects on the cytokine storm syndrome which may cause progression of the disease into more severe outcome.[18] Ivermectin is a drug that is used to manage parasitic infections with broad-spectrum effectivity and has been approved by The United States Food and Drug Administration (FDA). It has been long known for the treatment of onchocerciasis, strongyloidiasis, lymphatic filariasis and/or scabies.[19] Besides its potential as anti-parasitic agents, several articles have demonstrated the antiviral activity of ivermectin against various viruses.[20–22] Ivermectin has also been suggested to offer benefit in improving the outcomes from Covid-19 because of its action on prevention of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) proteins from entering the host cell nucleus in vitro.[23] Nonetheless, the evidence concerning the advantage of ivermectin, specifically in patients with SARS-CoV-2 infections, remains unclear. The objective of this meta-analysis is to explore the potential advantage of ivermectin to improve the outcomes of Covid-19 based on available randomized clinical trial studies.

2 | MATERIALS AND METHODS

2.1 | Eligibility criteria

The protocol of this study has been registered in PROSPERO (CRD42021230652). Included articles in this study are selected as potentially fulfilling the entry criteria: comply with the PICO framework (P: Population—Covid-19 patients; I: Intervention—ivermectin medications; C: Comparison or Control—a group of patients who did not receive ivermectin, only receive standard of care therapy or any other medications as control/placebo; O: Outcome—severe Covid-19, mortality, negative RT-PCR test results rate, time to negative RT-PCR test results, symptoms alleviations rate, time to symptoms alleviations and time to hospital discharge), randomized clinical trial articles were included, with the condition that the full-text paper was published. The exclusion criteria are any studies other than randomized clinical trials, studies reported other than in English language, studies focussing on the populations of young age (below 18 years old) and women during their pregnancy.

2.2 | Search strategy and study selection

The papers were searched systemically and obtained from PubMed, Europe PMC and ClinicalTrials.gov. Search terms used include 'ivermectin' OR 'stromectol' OR 'stromectal' OR 'sklice' OR 'ivomec' OR 'mectizan' AND 'SARS-CoV-2', OR 'coronavirus disease 2019' OR 'Covid-19' in a time range from 20th December 2019 until the present time (10th May 2021) with English-language restriction. The details regarding the search strategy used in this study are listed in Table 1. Studies evaluating the use of ivermectin therapy in patients with Covid-19, with a valid outcome of interest definition, were included in this study. Potential eligible articles searching was done by analysing the papers cited by authors of all identified studies. The search strategy was presented in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses diagram.

2.3 | Data extraction and quality assessment

Two authors performed the data extraction process. An extraction form was developed to list the essential information about the study and its population characteristic, ivermectin dose, time to ivermectin administration, control group medications, the number of patients receiving ivermectin and the control group, also each outcome of Covid-19 patients' proportion.

This study's outcomes of interest are rate of negative RT-PCR test results, rate of symptoms alleviations, time to negative RT-PCR test results, time to symptoms alleviations, time to hospital discharge, severe Covid-19 and mortality. The rate of negative RT-PCR test results was described by the number of patients who were converted from positive to negative RT-PCR test results at the end of follow-up. The rate of symptoms alleviations was described by the number of patients who have symptoms improvement or who are symptoms-free at the end of follow-up. Time to negative RT-PCR test results was defined by the time needed for the conversion from positive RT-PCR to negative RT-PCR test results. Time to symptoms alleviations was defined by the time needed for the patients' symptoms to be disappeared. Time to hospital discharge was defined by the duration needed for patients to be discharged from the hospital. Severe Covid-19 manifestation was the one having either of the mentioned features at the time of, or after, admission: (1) respiratory distress (≥30 breaths per min); (2) oxygen saturation at rest ≤93%; (3) ratio of the partial pressure of arterial oxygen (PaO2) to a fractional concentration of oxygen inspired air (FiO2) ≤300 mmHg; or (4) critical complication (respiratory failure, septic shock and or multiple

TABLE 1 Literature search strategy

Database	Keyword	Result
PubMed	('ivermectin'[MeSH Terms] OR 'ivermectin'[All Fields]) OR ('ivermectin'[MeSH Terms] OR 'ivermectin'[All Fields] OR 'stromectol'[All Fields]) OR stromectal[All Fields] OR sklice [All Fields] OR ('ivermectin'[MeSH Terms] OR 'ivermectin'[All Fields] OR 'ivomec'[All Fields]) OR ('ivermectin'[MeSH Terms] OR 'ivermectin'[All Fields] OR 'mectizan'[All Fields]) AND ('COVID-19'[All Fields] OR 'COVID-19'[MeSH Terms] OR 'COVID-19 Vaccines'[All Fields] OR 'COVID-19 Vaccines'[MeSH Terms] OR 'COVID-19 serotherapy'[All Fields] OR 'COVID-19 Nucleic Acid Testing'[All Fields] OR 'Covid-19 nucleic acid testing'[MeSH Terms] OR 'COVID-19 Serological Testing'[All Fields] OR 'Covid-19 serological testing'[MeSH Terms] OR 'COVID-19 Testing'[All Fields] OR 'Covid-19 testing'[MeSH Terms] OR 'SARS-CoV-2'[All Fields] OR 'sars-cov-2'[MeSH Terms] OR 'Severe Acute Respiratory Syndrome Coronavirus 2'[All Fields] OR 'NCOV'[All Fields] OR '2019 NCOV'[All Fields] OR (('coronavirus'[MeSH Terms] OR 'coronavirus'[All Fields] OR 'COV'[All Fields]) AND 2019/11/01[PubDate]: 3000/12/31 [PubDate]))	196
Europe PMC	'ivermectin' OR 'stromectol' OR 'stromectal' OR 'sklice' OR 'ivomec' OR 'mectizan' AND 'SARS-CoV-2', OR 'coronavirus disease 2019' OR 'Covid-19'	1350
ClinicalTrials.gov	'ivermectin' OR 'stromectol' OR 'stromectal' OR 'sklice' OR 'ivomec' OR 'mectizan' AND 'SARS-CoV-2', OR 'coronavirus disease 2019' OR 'Covid-19'	66

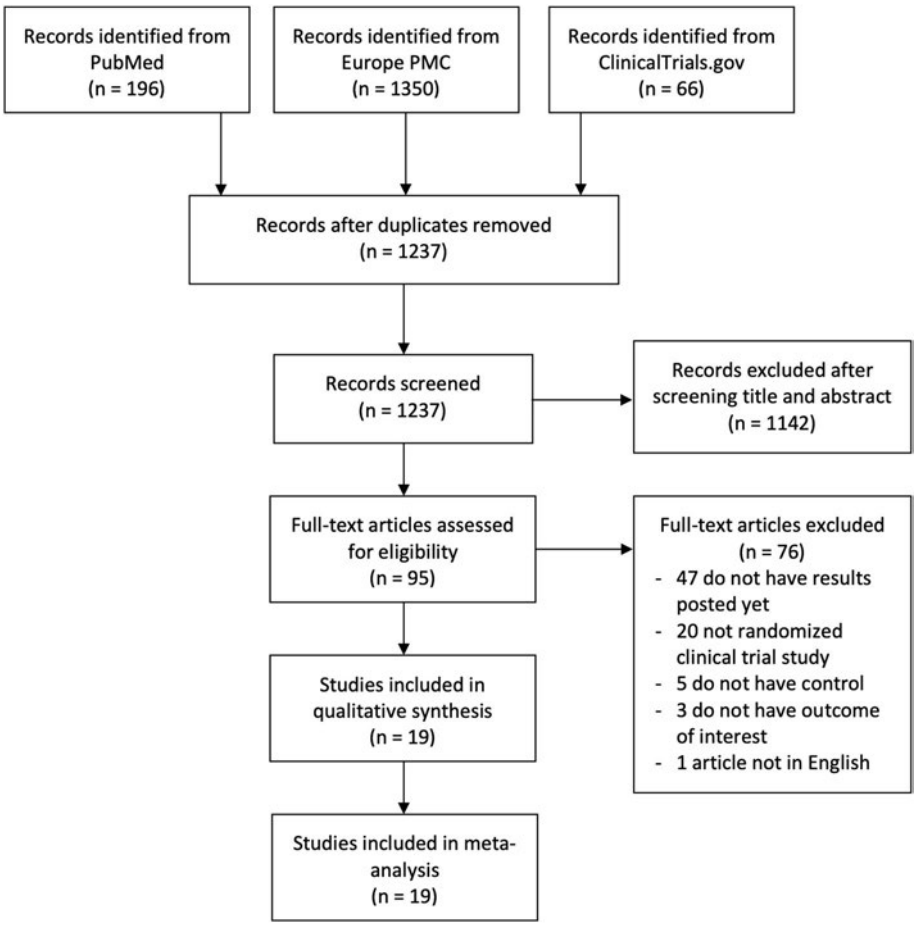


FIGURE 1 PRISMA diagram of the detailed process of selection of studies for inclusion in the systematic review and meta-analysis

organ dysfunction/failure) or intensive care unit admission. The total of dead patients due to Covid-19 was described as the mortality outcome.

The other two authors assessing each included studies' quality independently, using the Jadad scale assessment.[24] The random allocation, allocation concealment, blindness and withdrawals and

TABLE 2 Characteristics of included studies

Study	Sample size	Design	Overall age mean ± SD	Outcome	Ivermectin dose	Patient category	Control	Ivermectin versus control *n* (%)
Ahmed S et al. 2020[27]	72	Double-blind randomized clinical trial	42 ± 15.8	- Time to negative PCR - Symptoms alleviations rate - Time to hospital discharge	12 mg, once daily for 5 days	Mild to severe patients	Placebo	24 (33.3%) versus 24 (33.3%)
Babalola OE et al. 2021[28]	62	Double-blind randomized clinical trial	44.1 ± 14.7	- Time to negative PCR	Divided into two groups: (1): 6 mg, twice a week for 2 weeks; (2): 12 mg, twice a week for 2 weeks	Mild to moderate patients	Lopinavir/ritonavir for 2 weeks	42 (67.7%) versus 20 (32.3%)
Bukhari KHS et al. 2021[29]	86	Open-label randomized clinical trial	40.3 ± 12	- Negative PCR rate	12 mg, single dose	Mild to moderate patients	Standard of care treatment (vitamin C 500 mg once daily, vitamin D3 200,000 IU once weekly, Paracetamol 500 mg)	41 (47.6%) versus 45 (52.4%)
Chachar AZK et al. 2020[30]	50	Open-label Randomized clinical trial	41.8 ± 15.6	- Symptoms alleviations rate	Total three doses: 12 mg at start, 12 mg after 12 h and 12 mg after 24 h	Mild patients	Symptomatic treatment only	25 (50%) versus 25 (50%)
Chowdhury ATMM et al. 2020[31]	116	Open-label Randomized clinical trial	33.9 ± 14.2	- Negative PCR rate - Symptoms alleviations rate - Time to negative PCR - Time to symptoms alleviations	200 μg/kg, once daily for 10 days	Mild to moderate patients	Hydroxychloroquine 400 mg on the first day then 200 mg BID for 9 days + Azithromycin 500 mg daily for 5 days	60 (51.7%) versus 56 (48.3%)
Elgazzar A et al. 2020[32]	400	Open-label Randomized clinical trial	56.7 ± 18.4	- Severity - Mortality - Negative PCR rate - Symptoms alleviations rate	400 μg/kg, once daily for 4 days	Mild to moderate and severe patients	Hydroxychloroquine 400 mg BID for the first day then 200 mg BID for 5 days	200 (50%) versus 200 (50%)

FOI 26-3132 - Document 3

TABLE 2 (Continued)

Study	Sample size	Design	Overall age mean ± SD	Outcome	Ivermectin dose	Patient category	Control	Ivermectin versus control *n* (%)
				- Time to negative PCR - Time to hospital discharge				
Gonzalez JLB et al. 2021[33]	106	Double-blind randomized clinical trial	53.8 ± 16.9	- Mortality - Time to hospital discharge	12 mg, single dose in patients <80 kg and 18 mg, single dose in patients >80 kg	Severe patients	Calcium citrate as identical placebo	36 (33.9%) versus 37 (34.9%)
Hashim HA et al. 2020[34]	140	Open-label Randomized clinical trial	48.7 ± 8.6	- Severity - Mortality - Time to negative PCR - Time to hospital discharge	200 μg/kg, once daily for 2 days	Mild to moderate and severe-critical patients	Standard of care treatment (Azithromycin 250 mg/day for 5 days, vitamin C 1000 mg twice daily, zinc 75–125 mg/day, vitamin D3 5000 IU/day, acetaminophen 500 mg on need, dexamethasone 6 mg/day if needed, oxygen therapy if needed, and mechanical ventilation if needed)	70 (50%) versus 70 (50%)
Kishoria N et al. 2020[35]	32	Open-label randomized clinical trial	39.5 ± 15.4	- Negative PCR rate	12 mg, single dose	Mild to moderate patients	Hydroxychloroquine 400 mg BID + Paracetamol 500 mg as needed + Vitamin C 1 tablet BID	19 (59.3%) versus 13 (40.7%)
Lopez-Medina E et al. 2021[36]	398	Double-blind randomized clinical trial	37 ± 12.4	- Severity - Mortality - Symptoms alleviations rate - Time to symptoms alleviations	300 μg/kg/day for 5 days	Mild patients	Dextrose 5% as identical placebo	200 (50.2%) versus 198 (49.8%)
Mahmud R et al. 2020[37]	363	Double-blind randomized clinical trial	69.3 ± 9.6	- Severity - Mortality - Negative PCR rate - Symptoms alleviations rate	12 mg, once daily for 5 days	Mild to moderate patients	Standard of care treatment as an identical placebo (Paracetamol, vitamin D, oxygen if indicated, low molecular weight heparin, dexamethasone if indicated)	183 (50.4%) versus 180 (49.6%)
Mohan A et al. 2021[38]	125	Double-blind randomized	35.3 ± 10.5	- Severity		Mild to moderate patients	Identical placebo	80 (64%) versus 45

(Continues)

TABLE 2 (Continued)

Study	Sample size	Design	Overall age mean ± SD	Outcome	Ivermectin dose	Patient category	Control	Ivermectin versus control *n* (%)
		clinical trial		- Negative PCR rate - Time to negative PCR - Time to hospital discharge	Divided into two groups: (1): 12 mg, single dose; (2): 24 mg, single dose			(36%)
Niaee MS et al. 2020[39]	180	Double-blind randomized clinical trial	56 ± 16.2	- Mortality - Time to hospital discharge - Time to symptoms alleviations	Divided into several groups: (1): 200 μg/kg, single dose; (2): 200, 200 and 200 μg/kg in 1, 3 and 5 interval days; (3): 400 μg/kg, single dose; (4): 400, 200 and 200 μg/kg in 1, 3 and 5 interval days	Mild to severe patients	Standard of care treatment (Hydroxychloroquine 200 mg/kg, twice daily in an identical placebo)	120 (66.7%) versus 60 (33.3%)
Okumus N et al. 2021[40]	60	Open-label randomized clinical trial	62.2 ± 13	- Mortality - Negative PCR rate - Symptoms alleviations rate	200 mcg/kg/day for 5 days	Severe patients	Standard of care treatment (Hydroxychloroquine 2 × 400 mg loading dose followed by 2 × 200 mg for 5 days + favipiravir 2 × 1600 mg loading dose followed by 2 × 600 mg maintenance dose + azithromycin first day 500 mg followed by 4 days 250 mg/day) for a total of 5 days	30 (50%) versus 30 (50%)
Podder CS et al. 2020[41]	62	Open-label randomized clinical trial	39.1 ± 12	- Negative PCR rate - Time to symptoms alleviations	200 μg/kg, single dose	Mild to moderate patients	Standard of care treatment (Doxycycline 100 mg every 12 h for 7 days + symptomatic treatment)	32 (51.6%) versus 30 (48.4%)
Pott-Junior H et al. 2021[42]	31	Open-label randomized clinical trial	49.4 ± 14.6	- Severity - Negative PCR rate - Time to negative PCR	Divided into three groups: (1): 100 mcg/kg; (2): 200 mcg/kg; (3): 400 mcg/kg	Mild to severe patients	Standard of care treatment according to the latest recommendations on managing Covid-19	27 (87%) versus 4 (13%)
Ravikirti et al. 2021[43]	112	Double-blind randomized clinical trial	52.5 ± 14.7	- Severity - Mortality - Symptoms alleviations rate	12 mg, on days 1 and 2	Mild to moderate patients	Identical placebo tablets	55 (49.1%) versus 57 (50.9%)

FOI 26-3132 - Document 3

FOI 26-3732-Document 5

TABLE 2 (Continued)

Study	Sample size	Design	Overall age mean ± SD	Outcome	Ivermectin dose	Patient category	Control	Ivermectin versus control n (%)
Shahbaznejad L et al. 2021[44]	69	Double-blind randomized clinical trial	47.6 ± 22.1	- Time to hospital discharge - Time to symptoms alleviations	200 μg/kg, single dose	Moderate to severe patients	Hydroxychloroquine and/or lopinavir/ritonavir according to national protocols of Iran	35 (50.7%) versus 34 (49.3%)
Shouman W et al. 2020[45]	304	Open-label randomized clinical trial	39.7 ± 14.9	- Symptoms alleviations rate - Time to symptoms alleviations	According to body weight: - 40–60 kg: 15 mg - 60–80 kg: 18 mg - >80 kg: 24 mg Given once daily at days 1 and 3	Asymptomatic patients	Only observations	203 (66.7%) versus 101 (33.3%)

drop-outs of each study were evaluated. Then, studies were scored from zero to seven. A study ranked as a high-quality study if the score was >4.[25]

2.4 | Statistical analysis

Review Manager 5.4 (Cochrane Collaboration) software was used to perform the meta-analysis. Mantel–Haenszel's formula was done to obtain risk ratios (RR) and its 95% confidence interval (CI), while Inverse Variance method was used to obtain mean difference (MD) and its standard deviations (SD). The heterogeneity was assessed by using the I^2 statistic with a value of <25%, 26%–50% and >50% were considered as low, moderate and high degrees of heterogeneity, respectively. The results were considered significant when the two-tailed p-value was ≤0.05. The formula by Wan X et al. was used for meta-analytical pooling, if the available data were in medians and interquartile ranges to be converted to mean and SD.[26] The qualitative risk of publication bias was assessed with Begg's funnel plot analysis.

3 | RESULTS

3.1 | Study selection and characteristics

In electronic databases, 1612 studies were found. A total of 1237 records remained following the elimination of duplicates. By screening the titles/abstracts and matching the inclusion and exclusion criteria, 1142 studies were removed. Among the 95 evaluated full-text articles for its eligibility, 47 articles were excluded due to unposted results (still recruiting or withdrawn), 20 articles because the study designs are not randomized clinical trial study (non-randomized clinical trial, cross-sectional, observational studies, case-series), 5 articles because of no control/comparison group in the studies, 3 articles because of they do not mention the criteria of our outcome of interest and 1 article because the article was not in English. At last, the meta-analysis included 19 randomized clinical trial studies[27–45] with a total of 2768 Covid-19 patients (Figure 1). Amongst them, 10 were open-label randomized clinical trial studies, while the rest nine studies were double-blind randomized clinical trial studies. Table 2 presents the characteristic of the studies.

3.2 | Quality of study assessment

Jadad scale assessments were used to assess clinical trial studies (Table 3). Seven out of 19 included studies were graded 'high quality', while the other 12 studies were graded 'moderate quality'. To sum up, all papers were decent to be further analysed using meta-analysis.

FOI 26-3132 - Document 5

TABLE 3 Quality appraisal of studies included in the meta-analysis using Jadad scale assessment

Study	Random allocation	Concealment schemes	Blinding	Withdrawals and Drop-out	Total score	Interpretation
Ahmed S et al. 2020[27]	1	1	1	1	4	Moderate quality
Babalola OE et al. 2021[28]	1	1	1	1	4	Moderate quality
Bukhari KHS et al. 2021[29]	2	1	0	1	4	Moderate quality
Chachar AZK et al. 2020[30]	2	1	0	1	4	Moderate quality
Chowdhury ATMM et al. 2020[31]	2	1	0	1	4	Moderate quality
Elgazzar A et al. 2020[32]	1	1	0	1	3	Moderate quality
Gonzalez JLB et al. 2021[33]	1	1	2	1	5	High quality
Hashim HA et al. 2020[34]	0	1	1	1	3	Moderate quality
Kishoria N et al. 2020[35]	2	1	0	1	4	Moderate quality
Lopez-Medina E et al. 2021[36]	2	2	2	1	7	High quality
Mahmud R et al. 2020[37]	2	1	2	1	6	High quality
Mohan A et al. 2021[38]	2	1	2	1	6	High quality
Niaee MS et al. 2020[39]	2	2	2	1	7	High quality
Okumus N et al. 2021[40]	1	1	0	1	3	Moderate quality
Podder CS et al. 2020[41]	0	1	1	1	3	Moderate quality
Pott-Junior H et al. 2021[42]	2	2	0	1	5	High quality
Ravikirti et al. 2021[43]	2	2	1	1	6	High quality
Shahbaznejad L et al. 2021[44]	0	1	1	1	3	Moderate quality
Shouman W et al. 2020[45]	1	1	0	1	3	Moderate quality

Note: We used Jadad scale to assess the included studies. Points were determined as follows: (I) Random allocation: computer-generated random numbers, 2 points; not described, 1 point; inappropriate method, 0 point. (II) Allocation concealment: central randomization, sealed envelopes or similar, 2 points; not described, 1 point; inappropriate or unused, 0 point. (III) Blindness: identical placebo tablets or similar, 2 point; inadequate or not described, 1 point; inappropriate or no double blinding, 0 point. (IV) Withdrawals and drop-outs: numbers and reasons are described, 1 point; not described, 0 point. The Jadad scale score ranges from 1 to 7; higher score indicates better RCT quality. If a study had a modified Jadad score >4 points, it was considered to be of high quality; if the score was 3–4 points, it was moderate quality; and if the score was <3 points, it was low quality.

3.3 | Ivermectin and outcomes

3.3.1 | Severe Covid-19

Eight studies (n = 1638) reported on the severe Covid-19 outcome. Our pooled analysis showed that ivermectin administration was associated with reduction of severe Covid-19 outcome (RR 0.43 [95% CI 0.23–0.81], $p = 0.008$, $I^2 = 65\%$, random-effect modelling; Figure 2a).

3.3.2 | Mortality

Eight studies (n = 1726) reported on the mortality outcome. Our pooled analysis showed that ivermectin administration was associated with reduction of mortality from Covid-19 (RR 0.31 [95% CI 0.15–0.62], $p = 0.001$, $I^2 = 40\%$, random-effect modelling; Figure 2b).

3.3.3 | Negative RT-PCR test results rate

Nine studies (n = 1205) reported on the negative RT-PCR test results rate outcome. Our pooled analysis showed that ivermectin administration was associated with higher rate of negative Covid-19 RT-PCR test results (RR 1.23 [95% CI 1.01–1.51], $p = 0.04$, $I^2 = 91\%$, random-effect modelling; Figure 2c).

3.3.4 | Time to negative RT-PCR test results

Six studies (n = 782) reported on the time to negative RT-PCR test results outcome. Our pooled analysis showed that ivermectin administration was associated with shorter time to negative Covid-19 RT-PCR test results (MD −3.29 (95% CI −5.69, −0.89), $p = 0.007$, $I^2 = 96\%$, random-effect modelling; Figure 2d).

3.3.5 | Symptoms alleviations rate

Eight studies (n = 1535) reported on the symptoms alleviations rate outcome. Our pooled analysis showed that ivermectin administration was associated with higher rate of symptoms alleviations (RR 1.23 [95% CI 1.03–1.46], $p = 0.02$, $I^2 = 85\%$, random-effect modelling; Figure 2e).

FOI 26-3132 - Document 5

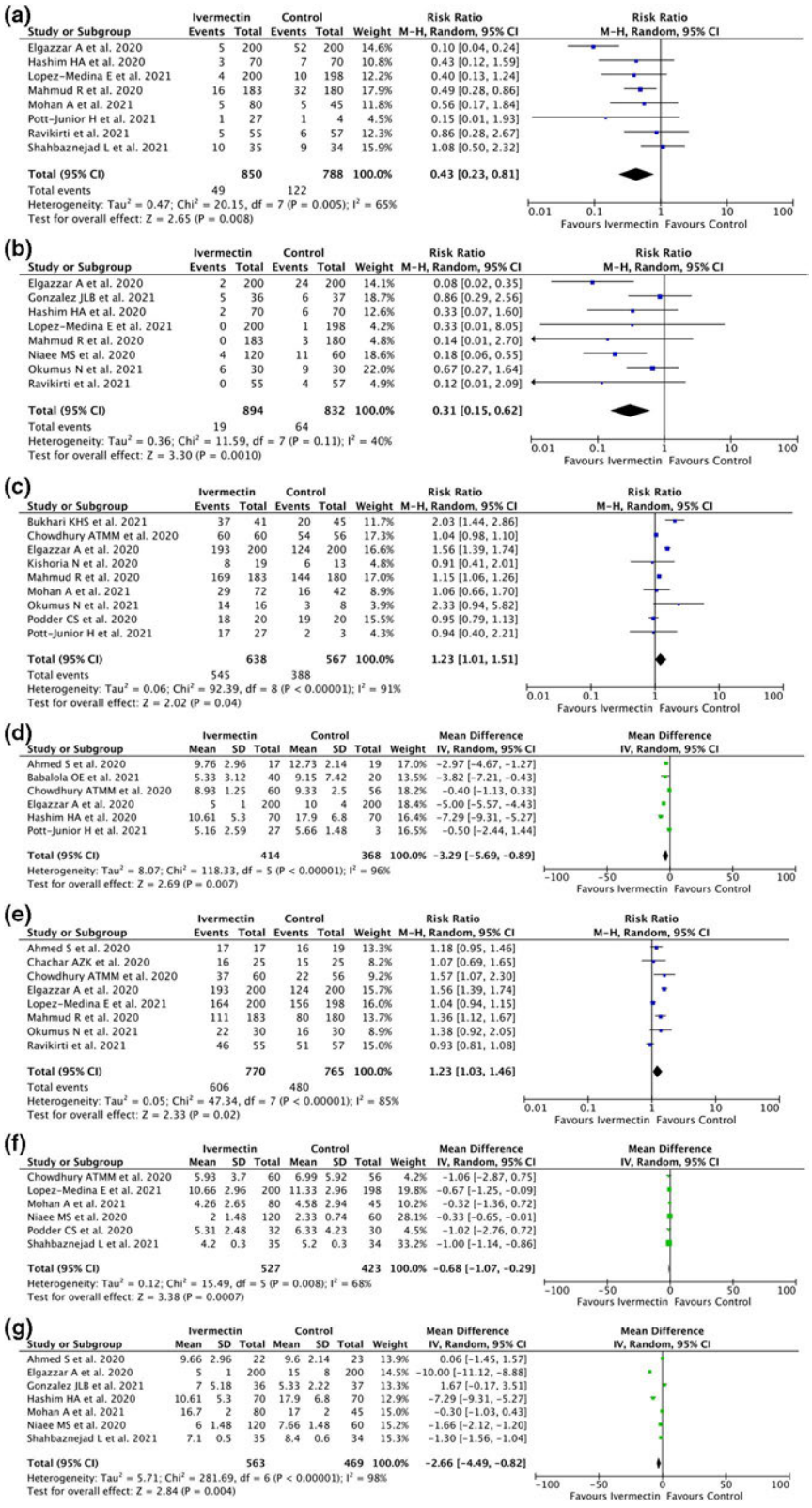


FIGURE 2 Forest plot that demonstrates the association of ivermectin administration with severe Covid-19 (a), mortality (b), negative RT-PCR test results rate (c), time to negative RT-PCR test results (d), symptoms alleviations rate (e), time to symptoms alleviations (f) and time to hospital discharge (g) outcomes

FOI 26-3132 - Document 5

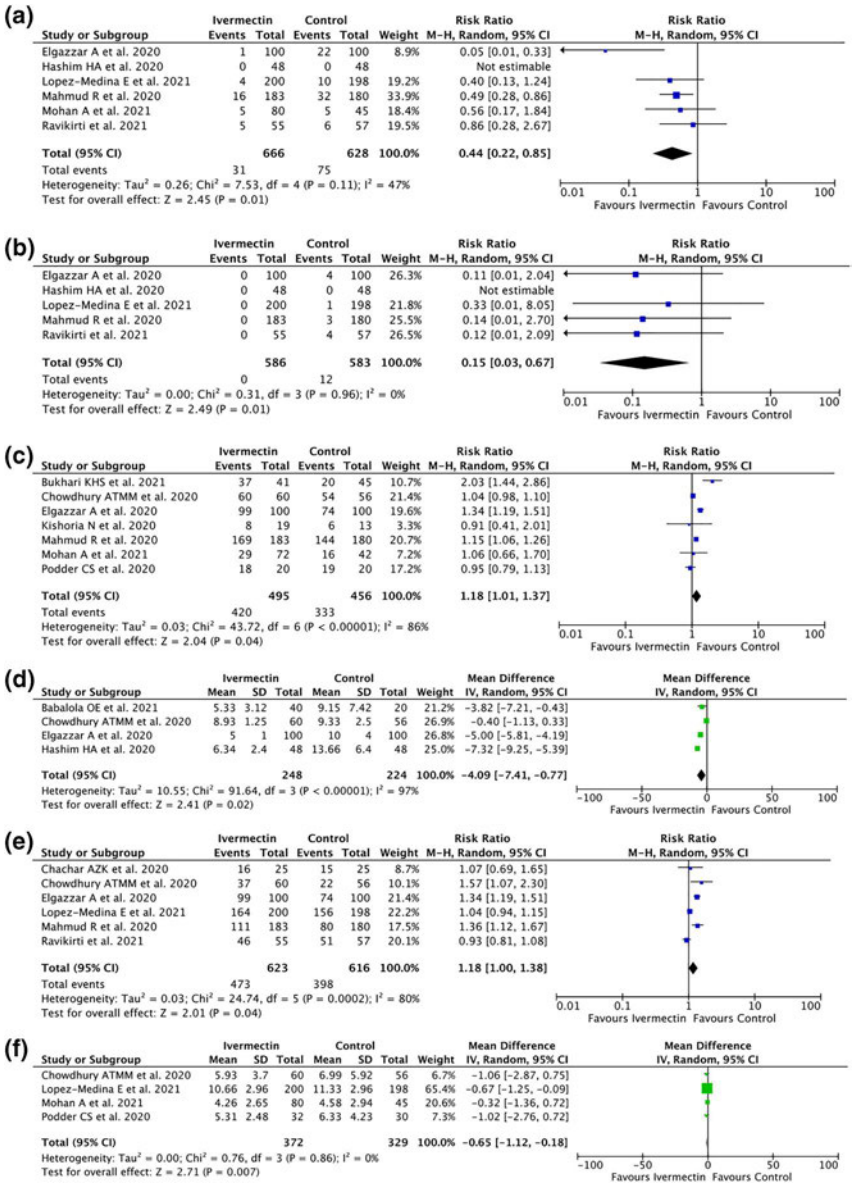


FIGURE 3 Subgroup analysis in mild to moderate patients that demonstrates the association of ivermectin administration with severe Covid-19 (a), mortality (b), negative RT-PCR test results rate (c), time to negative RT-PCR test results (d), symptoms alleviations rate (e) and time to symptoms alleviations (f) outcomes

3.3.6 | Time to symptoms alleviations

Six studies (n = 950) reported on the time to symptoms alleviations outcome. Our pooled analysis showed that ivermectin administration was associated with shorter time to symptoms alleviations (MD −0.68 (95% CI −1.07, −0.29), p = 0.0007, I^2 = 68%, random-effect modelling; Figure 2f).

3.3.7 | Time to hospital discharge

Seven studies (n = 1,032) reported on the time to hospital discharge outcome. Our pooled analysis showed that ivermectin administration was associated with shorter time to hospital discharge (MD −2.66 (95% CI −4.49, −0.82), p = 0.004, I^2 = 98%, random-effect modelling; Figure 2g).

3.4 | Subgroup analysis

Subgroup analysis revealed that ivermectin administration in mild to moderate patients showed a lower RR, therefore was associated with lower severity (n = 1294; RR 0.44 [95% CI 0.22–0.85], p = 0.01, I^2 = 47%, random-effect modelling; Figure 3a) and mortality (n = 1169; RR 0.15 [95% CI 0.03–0.67], p = 0.01, I^2 = 0%, random-effect modelling; Figure 3b) outcomes when compared with severity (n = 313; RR 0.41 [95% CI 0.10–1.59], p = 0.20, I^2 = 83%, random-effect modelling; Figure 4a) and mortality (n = 377; RR 0.41 [95% CI 0.16–1.04], p = 0.06, I^2 = 58%, random-effect modelling) outcomes (Figure 4b) in severe patients.

On the other side, subgroup analysis also revealed that ivermectin administration in severe patients showed a higher RR and higher MD in terms of negative RT-PCR test results rate (n = 224; RR 1.90 [95% CI 1.56–2.31], p < 0.00001, I^2 = 0%, random-effect

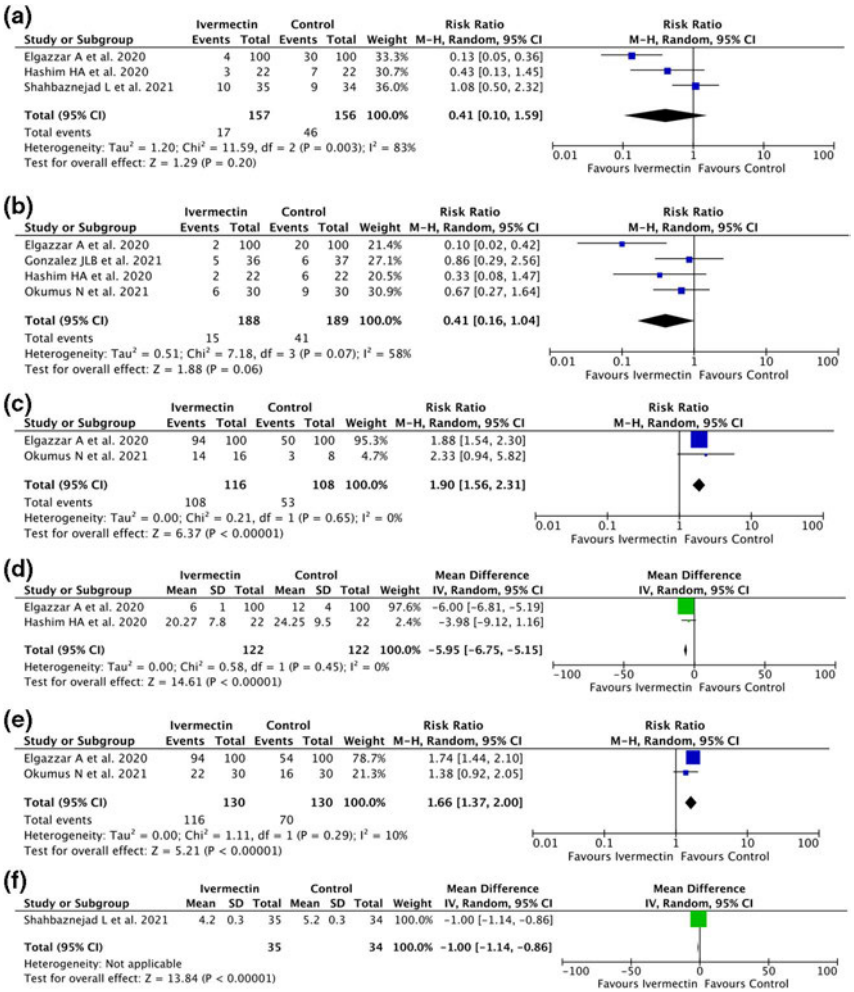


FIGURE 4 Subgroup analysis in severe patients that demonstrates the association of ivermectin administration with severe Covid-19 (a), mortality (b), negative RT-PCR test results rate (c), time to negative RT-PCR test results (d), symptoms alleviations rate (e) and time to symptoms alleviations (f) outcomes

modelling; Figure 4c), time to negative RT-PCR test results (n = 244; MD −5.95 (95% CI −6.75, −5.15), p < 0.00001, I^2 = 0%, random-effect modelling; Figure 4d), symptoms alleviations rate (n = 260; RR 1.66 [95% CI 1.37–2.00], p < 0.0001, I^2 = 10%, random-effect modelling; Figure 4e) and time to symptoms alleviations (n = 69; MD −1.00 [95% CI −1.14, −0.86), p < 0.00001, I^2 = 0%, random-effect modelling; Figure 4f) when compared with those outcomes in mild to moderate patients (negative RT-PCR test results rate, n = 951 [RR 1.18 (95% CI 1.01–1.37), p = 0.04, I^2 = 86%, random-effect modelling; Figure 3c], time to negative RT-PCR test results, [n = 472; MD −4.09 (95% CI −7.41, −0.77), p = 0.02, I^2 = 97%, random-effect modelling; Figure 3d], symptoms alleviations rate [n = 1239; RR 1.18 (95% CI 1.00–1.38), p = 0.04, I^2 = 80%, random-effect modelling; Figure 3e] and time to symptoms alleviations [n = 701; MD −0.65 (95% CI −1.12, −0.18), p = 0.007, I^2 = 0%, random-effect modelling; Figure 3f]).

3.5 | Publication bias

Funnel plot analysis showed a relatively symmetrical inverted-plot for the negative RT-PCR test results rate (Figure 5c), time to negative RT-PCR test results (Figure 5d), symptoms alleviations rate (Figure 5e), time to symptoms alleviations (Figure 5f) and time to hospital discharge (Figure 5g), showing no indication of publication bias. Funnel plot analysis showed an asymmetrical inverted-plot for the severe Covid-19 (Figure 5a) and mortality outcome (Figure 5b), showing some indication of publication bias. However, because the number of included studies in each outcomes are fewer than 10 studies, the funnel plots and statistical tests for detecting publication bias are not much reliable when compared with whenever there are more than 10 included studies in each outcomes.[46,47]

4 | DISCUSSION

According to our pooled analysis, ivermectin was discovered to have an association with a higher negative RT-PCR test results rate, shorter time to negative RT-PCR test results, higher symptoms alleviations rate, shorter time to symptoms alleviations, shorter time to hospital discharge and reduction in the severity and mortality from Covid-19. Our subgroup analysis also showed that the benefits of ivermectin therapy in reducing the severity and mortality outcomes from Covid-19 were more prominent when administered into mild to

FOI 26-3132 - Document 5

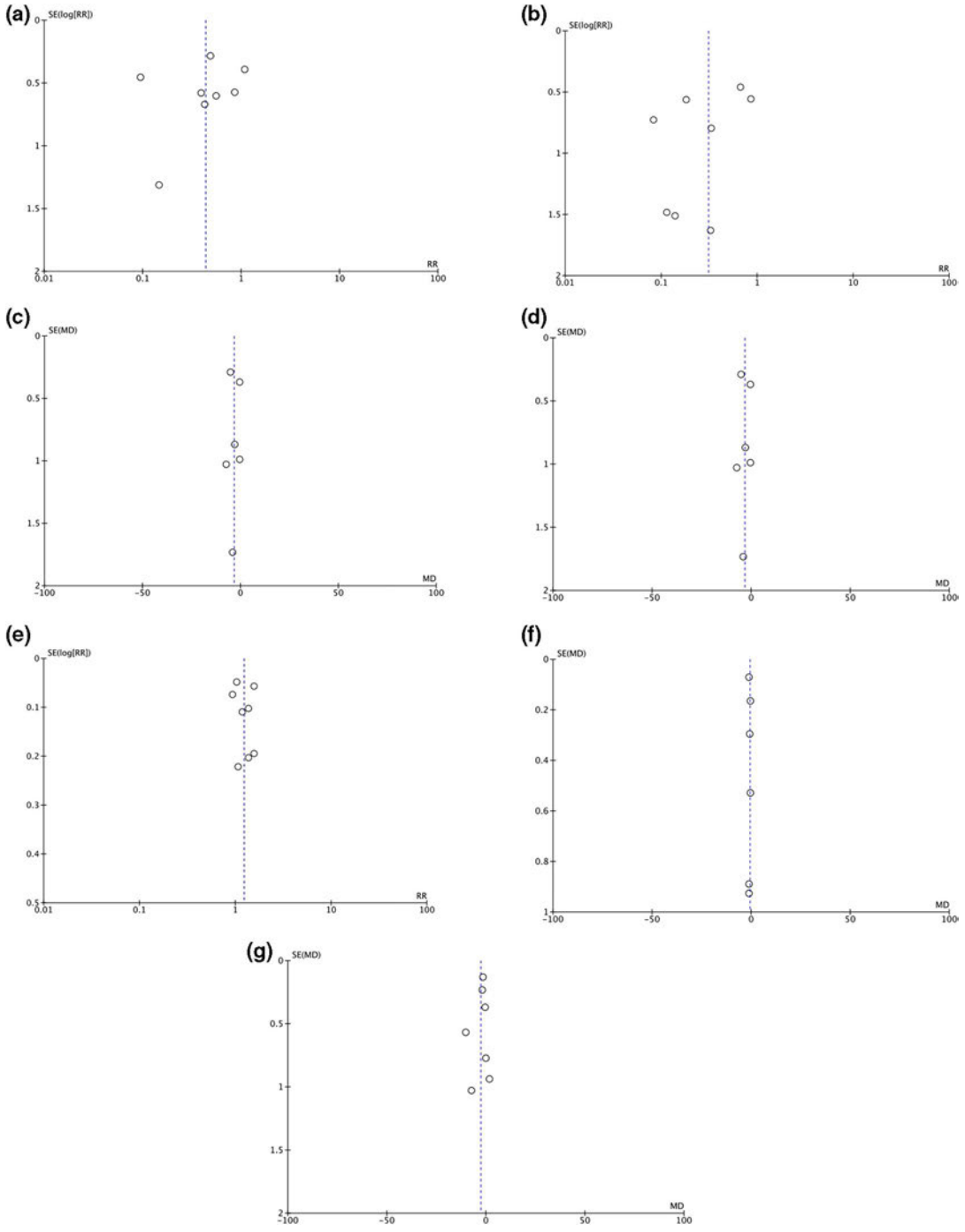


FIGURE 5 Funnel plot analysis for the association of ivermectin administration with severe Covid-19 (a), mortality (b), negative RT-PCR test results rate (c), time to negative RT-PCR test results (d), symptoms alleviations rate (e), time to symptoms alleviations (f), and time to hospital discharge (g) outcomes

moderate patients, compared with in severe patients. On the other side, the benefits of ivermectin therapy in increasing negative RT-PCR test results rate, shortening time to negative RT-PCR test results, increasing symptoms alleviations rate and shortening time to symptoms alleviations were more apparent in severe patients when compared with in mild to moderate patients.

A few arguments might explain these findings. The sequestration of the SARS-CoV-2 viral nucleocapsid protein (NCP) into the host nucleus through the nuclear-pore-complex is a vital step in viral pathogenesis and defence against host immune response.[48] Ivermectin can selectively inhibit the host importin α/β transporter protein which decreases translocation (shuttling) of SARS-CoV-2 NCP from the cytoplasm to the nucleus, alteration of NCP distribution will lead to viral propagation disruption and survival.[23] The in vitro study by Caly et al.[23] has proved that giving ivermectin in one dose was able to reduce the viral RNA load by 99.98% at 48 h and

replication of an Australian isolate of SARS-CoV-2 in Vero/hSLAM cells by 5000-folds. Therefore, it has potent effects in altering disease progression and spread. These in vitro findings were further supported with the results from a double-blind, placebo-controlled, randomized clinical trial study, showing that patients who received ivermectin 400 μg/kg single dose have a lower median viral load at Day 4 (161,000 vs. 493,500 copies/ml) and Day 7 post-treatment (1018 vs. 23,550 copies/ml). The differences were found, rising from a threefold decrease on the fourth day to about 18-fold lower on the seventh day when compared with placebo.[49] Second, the pathophysiologic process which underlies severe Covid-19 involves hyperinflammatory response and accumulation of cytokines, called a cytokine storm. A meta-analysis study has demonstrated that severe Covid-19 patients tend to produce higher cytokine levels such as interleukin-6 (IL-6), IL-8, IL-10 and tumour necrosis factor-α (TNF-α), in comparison to non-severe cases.[50] On the other side, an anti-inflammatory effect was also demonstrated in ivermectin, both in vivo and in vitro studies. Ivermectin can reduce the IL-1, IL-6, TNF-α production and suppressing lipopolysaccharide-induced nuclear factor-kappa B translocation.[51] The suppression of mucus due to hypersecretion in the respiratory tract, the reduction of immune cell recruitment, and a decrease in the production of cytokines and immunoglobulin E/immunoglobulin G1 in bronchoalveolar lavage of experimental mice, were found as a consequence of 2 mg/kg of ivermectin administration.[52] These findings suggest that ivermectin has an anti-inflammatory effect on the lung tissue, besides at the systemic level, which might help to reduce the severity and prevent mortality from Covid-19.

This study has several limitations. First, significant heterogeneities were found on most of the outcomes of interests included in this study. This was probably caused by the difference in the given ivermectin doses and the medications used as a standard of care or placebo. Second, the total number of patients included in this study was relatively small because at this time, ivermectin is still considered as new repurposed drug for Covid-19 where early trials still show conflicting results and there is still no meta-analysis study to support its efficacy, therefore it may be difficult to collect the participants and receiving their consent to participate in the trials. Third, we include some pre-print studies to minimize the risk of publication bias; however, the authors have made exhaustive efforts to ensure that only sound studies were included, and we expect that most of those studies currently available in pre-print form will eventually be published and that we will identify them through ongoing electronic literature surveillances. We hope that this study can give further insight into the management of Covid-19 patients.

5 | CONCLUSION

Our meta-analysis of randomized clinical trial studies indicates that ivermectin administration had an association with favourable outcomes of Covid-19, compromising of higher rate of negative RT-PCR test results, shorter time to negative RT-PCR test results, higher rate of symptoms alleviations, shorter time to symptoms alleviations, shorter time to hospital discharge, reduction in the severity and mortality rate from Covid-19. This study suggests that ivermectin may be the potential therapeutic agents for the managements of Covid-19 to give better outcomes for the patients. However, more randomized clinical trial studies are still necessary and encouraged to be done for confirming the results of our study. Finally, ivermectin should be considered as an essential drug for future Covid-19 therapy models.

ACKNOWLEDGEMENTS

None.

CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Conceptualization, methodology, formal analysis, data curation, writing-original draft, visualization, writing-review and editing: Timotius Ivan Hariyanto. Conceptualization, methodology, formal analysis, data curation, writing-original draft, writing-review and editing: Devina Adella Halim. Conceptualization, validation, supervision, writing-review and editing: Jane Rosalind. Conceptualization, validation, supervision, writing-review and editing: Catherine Gunawan. Conceptualization, validation, supervision, writing-review and editing: Andree Kurniawan.

DATA AVAILABILITY STATEMENT

Data analysed in this study were a re-analysis of existing data, which are openly available at locations cited in the reference section.

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How to cite this article: Hariyanto TI, Halim DA, Rosalind J, Gunawan C, Kurniawan A. Ivermectin and outcomes from Covid-19 pneumonia: A systematic review and meta-analysis of randomized clinical trial studies. *Rev Med Virol*. 2021; e2265. https://doi.org/10.1002/rmv.2265

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Controlled randomized clinical trial on using Ivermectin with Doxycycline for treating COVID-19 patients in Baghdad, Iraq

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Registered in clinicaltrials.gov: NCT04591600

Abstract

Objectives: COVID-19 patients suffer from the lack of curative therapy. Hence, there is an urgent need to try repurposed old drugs on COVID-19. **Methods**: Randomized controlled study on 70 COVID-19 patients (48 mild-moderate, 11 severe, and 11 critical patients) treated with 200ug/kg PO of Ivermectin per day for 2-3 days along with 100mg PO doxycycline twice per day for 5-10 days plus standard therapy; the second arm is 70 COVID-19 patients (48 mild-moderate and 22 severe and zero critical patients) on standard therapy. The time to recovery, the progression of the disease, and the mortality rate were the outcome-assessing parameters. **Results:** among all patients and among severe patients, 3/70 (4.28%) and 1/11 (9%), respectively progressed to a more advanced stage of the disease in the Ivermectin-Doxycycline group versus 7/70 (10%) and 7/22 (31.81%), respectively in the control group ($P>0.05$). The mortality rate was 0/48 (0%), 0/11 (0%), and 2/11 (18.2%) in mild-moderate, severe, and critical COVID-19 patients, respectively in Ivermectin-Doxycycline group versus 0/48 (0%), and 6/22 (27.27%) in mild-moderate and severe COVID-19 patients, respectively in standard therapy group ($p=0.052$). Moreover, the mean time to recovery was 6.34, 20.27, and 24.13 days in mild-moderate, severe, and critical COVID-19 patients, respectively in Ivermectin-Doxycycline group versus 13.66 and 24.25 days in mild-moderate and severe COVID-19 patients, respectively

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in standard therapy group (P<0.01). **Conclusions:** Ivermectin with doxycycline reduced the time to recovery and the percentage of patients who progress to more advanced stage of disease; in addition, Ivermectin with doxycycline reduced mortality rate in severe patients from 22.72% to 0%; however, 18.2% of critically ill patients died with Ivermectin and doxycycline therapy. Taken together, the earlier administered Ivermectin with doxycycline, the higher rate of successful therapy.

Keywords: Ivermectin, Doxycycline, COVID-19, Coronavirus, SARS-CoV-2

Introduction

Since December 2019, the world has been facing unprecedented health caused by global spread of a novel coronavirus, SRARS-CoV-2 which causes respiratory, and multi-organ viral infection, called COVID-19. The pandemic of COVID-19 resulted in 35 million infections and more than one million deaths till the date of writing this article [1]. Almost all patients start as mild-moderate disease; however, about 15% of them progress within 5-14 days post infection to a more advanced stage of the disease, being severe and critical patients [2]. The highest risk patients have been shown to be elderly, obese, diabetic, immunosuppressed, or those with cardiovascular diseases [3, 4].

The main problem of COVID-19, there is no curative therapy till now. And the vaccine development takes time. Even after developing a successful vaccine, SARS-CoV-2 seems to induce a short lasting humeral immunity, 3-12 months only. Therefore, in the coming years when COVID-19 will be converted to seasonal endemic viral infection, a reliable therapy is still needed along with working vaccines [5].

From December 2019 till September 2020, no single drug was found to be a silver bullet for COVID-19. COVID-19 starts as respiratory viral infection but in some patients progress to severe viral pneumonia and very dangerous immune deregulation condition called cytokine storm which if is not promptly treated, it might result in acute respiratory distress syndrome (ARDS), multi-organ failure, lethal

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coagulopathies and death [2, 4, 6]. Therefore, potent antiviral therapy and immunomodulatory therapy for COVID-19 patients are desperately needed.

Ivermectin reduced viral load of SARS-CoV-2 *in vitro* by 5000 folds within 48h [7]. Moreover, several previous reports revealed antiviral activity of Ivermectin on Dengue HIV, Yellow fever, West Nile, Hendra, Newcastle, and Zika viruses [8-10]. Furthermore, several observational studies and real-world clinical practice showed that Ivermectin is effective in treating COVID-19 patients at both mild-moderate and severe phases of the disease [11-14]; accordingly, it is thought that Ivermectin might possess antiviral as well as immunomodulatory activity [12-14]. Ivermectin is most probably a host-specific antiviral drug and it acts as a specific inhibitor of importin α/β-mediated nuclear import inhibiting replication of several viruses such as HIV-1, Zika and dengue viruses [10]. It is thought that Ivermectin might inhibit SARS-CoV-2 using the same mechanism [7]. In addition, there have been several reports revealed that Ivermectin acts as anti-inflammatory and immunomodulatory agent and it can curb over-reacting innate and cellular immune responses [8, 15]. This explains how Ivermectin could alleviate symptoms of COVID-19 patients at viral replication phase (the first 7-10 days of infection) as well as the later hyperinflammatory phase [7-10].

Doxycycline is a broad spectrum antibiotic with reported antiviral activities on several viruses including SARS-CoV-2 [16-18]. The mechanism of the antiviral effects of tetracycline derivatives might be due to transcriptional upregulation of intracellular zinc finger antiviral protein which serves for encoding genes in host cells [19]. Doxycycline acts as an ionophore for zinc facilitating zinc entry into human cells and increasing cytoplasmic zinc concentration; high intracellular zinc levels are inhibitory to the replication of RNA viruses in cytoplasm of the cells by inhibiting RNA-depndent RNA polymerase enzyme [20]. Moreover, doxycycline has immune dampening effect making it useful to ease over-reacting immune systems [21].

In an attempt to find an effective therapy to COVID-19 patients, the current clinical trial was set up to test the combinational therapy of Ivermectin and Doxycycline in treating COVID-19 patients at different stages of the disease.

Patients and methods

Patients

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One hundred forty (140) COVID-19 patients at different stages of the disease were included in this study. Half of them (70 patients) received Ivermectin with Doxycycline and standard care while the other half (70 patients) received the standard care only. The patients were recruited in Alkarkh and Alforat hospitals in Baghdad city in the duration from July 1st to September 30th. The recruited patients were either outpatients or inpatients, according to the severity of the disease. Mild-moderate patients were outpatients while severe and critical patients were all inpatients. All of the recruited COVID-19 patients were diagnosed by clinical, radiological and laboratory PCR testing. Alike, recovery of COVID-19 patients was based on the disappearance of symptoms, clearance of radiological chest x-ray or Ct-scans, and getting negative PCR results.

The classification of COVID-19 patients to mild-moderate, severe, and critical was carried out according to the WHO guidelines. Ivermectin-Doxycycline group consisted of 48 mild-moderate, 11 severe and 11 critical patients while the control group consisted of 48 mild-moderate and 22 severe patients. For ethical basis, no critical patient recruited in this study was allocated to the control group; all of critical patients were allocated to the Ivermectin-Doxycycline group. The classification of the recruited patients was based on the stage of the disease at the first day of recruitment; the designated therapy of the current study was initiated at the first day of recruitment. Inclusion criteria of the patients enrolled in the clinical trial were those who were symptomatic for no more than three days for mild-moderate cases, no more than two days after being severe cases, and no more than one day after being critical cases. The purpose behind this was to assess Ivermectin-Doxycycline therapy versus standard care therapy at the beginning of each stage of the disease. The recruited patients were monitored till recovery or death.

The present study was approved by the ethical and scientific committee in Baghdad-Alkarkh General Directorate of under the approval number BKH-CT-016.

Randomization of patients

COVID-19 patients were randomly allocated to one of the study groups depending on a simple method. Patients recruited at dates with odd number were allocated to

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Ivermectin-Doxycycline group while other patients were allocated to the control group. Inside each group, maximal limit of 48 mild-moderate patients and 22 severe and/or critical patients were allowed. The randomization process as well as the patients records for disease progression, recovery, and clinical or laboratory testing were supervised by the health authority of Alkarkh Health General Directorate in Baghdad city.

Protocols of therapy

Ivermectin-Doxycycline group

Ivermectin 200ug/kg PO per day for two days, and in some patients who needed more time to recover, a third dose 200ug/kg PO per day was given 7 days after the first dose. Doxycycline 100mg capsule PO every 12h per day was given for 5-10 days, based on the clinical improvement of patients. In addition, standard care was given to the patients of Ivermectin-Doxycycline group based on the clinical condition of each patient.

Control group: The patients in this group received only standard care which included all or some of the following, according to the clinical condition of each patient.

Standard care

- Acetaminophen 500mg on need
- Vitamin C 1000mg twice/ day
- Zinc 75-125 mg/day
- Vitamin D3 5000IU/day
- Azithromycin 250mg/day for 5 days
- Oxygen therapy/ C-Pap if needed
- Dexamethazone 6 mg/day or methylprednisolone 40mg twice per day, if needed
- Mechanical ventilation, if needed

Outcome-assessing parameters of the disease progression or recovery

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Three parameters used in the present study were to assess the disease progression or recovery in COVID-19 patients who received standard care only compared to patients who received standard care with Ivermectin and Doxycycline therapy. These three parameters are:

1. Time to recovery, if any. It is the time between taking therapy till recovery.
2. Percentage of patients who progress to a more advanced stage of the disease after at least 3 days of giving therapy. For example, a patient was recruited as mild-moderate; after 3 days from starting therapy, the patient progressed to severe stage; such patient is considered a progressing patient even though she or he was under treatment.
3. Mortality rate among mild-moderate, severe, or critical patients in Ivermectin-Doxycycline group versus those in control group.

Statistical analysis

Data was processed according to the normality tests results; parametric data were represented with mean values and non-parametric data were represented with median values. Percentage of mortality rate was calculated for each group of the study. Odds ratio and chi-square were used to test the strength and significance of association. P values less than 0.05 was considered significant

Results

Patients characteristics

Mean age of the recruited patients was 48.7±8.6 year with range 16 to 86 year; patients in Ivermectin-Doxycycline and control groups were age- and sex- matched. Mean age of Ivermectin-Doxycycline group was 50.1±9.3 year with 53% males and 47% females while mean age of control group patints was 47.2±7.8 year with 51% male and 49% females ($P>0.05$). In both groups, the median post-infection day for starting therapy was 3 days in mild-moderate, 7 days in severe, and 8.5 days in critical cases. The mean weight of Ivermectin-Doxycycline and control patients was 79.6±13.2 kg and 71.5±11.9 Kg, respectively ($P>0.05$).

Time to recovery

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The time to recovery was shown to be significantly reduced in the Ivermectin-Doxycycline compared to the control group; mean recovery time in Ivermectin-Doxycycline group was 10.61± 5.3 days versus mean recovery time in control group, 17.9±6.8 days ($P<0.05$). Hence, using Ivermectin along with Doxycycline reduced mean time to recovery up to 7 days. By analyzing the mean time to recovery in mild-moderate, severe, or critical patients in each group, it was shown that the mean time to recovery in Ivermectin-Doxycycline group was 6.34±2.4, 20.27±7.8, 19.77±9.2 days, respectively versus 13.66±6.4, 24.25±9.5 days, in control group, respectively ($P<0.01$). Accordingly, Ivermectin-Doxycycline reduced recovery time about 7.32 days in mild-moderate, and 3.98 or roughly 4 days in severe patients (Table 1).

Progression of the disease

The rate of progression of the disease, or the deterioration of the clinical condition of the patients despite of taking standard care with/without Ivermectin-doxycycline therapy, was shown to be varied between the two groups studied. At beginning, no single mild-moderate patient in both groups progressed to a more advanced stage of the disease. For severe COVID-19 patients, 1/11 (9%) in Ivermectin-Doxycycline group versus 7/22 (31.81%) in control group progressed to more advanced stage of the disease, namely being classified as critical cases ($P>0.05$). Thus, Ivermectin-Doxycycline protocol was shown to lower progression of the disease in severe patients if given within the first two days of the severe stage of the disease. For critical patients, ethically, critical patients were not included in the control group as critical patients need to receive all possible medications for saving their lives; hence, it was not possible to compare the rate of progression of the disease in critical patients between the two studied groups (Table 1).

Mortality rate

The mortality rate was shown to be 0/48 (0%) in mild-moderate patients in both groups. Nevertheless, the mortality rate was diminished to 0/11 (0%) in Ivermectin-Doxycycline group compared to 6/22 (27.27%) ($P=0.052$). For critical patients, Ivermectin-Doxycycline did not prevent death in those patients as mortality rate was shown to be 2/11 (18.2%), (Table 1). No critical patients were included in the control group to compare with; however, it is obvious that 18.2% of death in critical COVID-19 patients received Ivermectin-Doxycycline is much lower than the death rate in

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critical cases of COVID-19 in Iraq that might reach above 50% (based on real-world data not official published data).

Table 1: Parameters of the study outcomes between Ivermectin-Doxycycline and control groups

Outcome parameter	Subgroup of patients	Ivermectin-Doxycycline	Control	P value
Time to recovery (day)	Total	10.61± 5.3	17.9±6.8	<0.0001
	Mild-moderate	6.34±2.4	13.66±6.4	<0.0001
	severe	20.27±7.8	24.25±9.5	0.29
	critical	19.77±9.2	/	
Rate of progression of disease number/total (%)	Total	3/70 (4.28%)	7/70 (10%)	0.19 (OR*=0.4, P=0.2)
	Mild-moderate	0/48 (0%)	0/48 (0%)	1
	severe	1/11 (9%)	7/22 (31.81%)	0.15 (OR=0.21, P=0.17)
	critical	2/11 (18.2%)	/	
Mortality rate	Total	2/70 (2.85%)	6/70 (7.14)	0.14 (OR=0.31, P=0.16)
	Mild-moderate	0/48 (0%)	0/48 (0%)	1
	severe	0/11 (0%)	6/22 (27.27%)	0.052 (OR=0.11, P=0.14)
	critical	2/11 (18.2%)	/	

OR*: Odds ratio

Discussion

Finding an effective therapy for COVID-19 is an ultimate goal for health bodies all over the world. The problem of the standard care for COVID-19 patient is not curative; however, the current situation is much better than the first months of the pandemic , after introducing steroid therapy for severe/critical patients and high doses of vitamin D3, vitamin C and Zinc for mild-moderate cases [22]. COVID-19 is a multiphasic disease starting with virus replicative phase lasting for 7-10 days then, in some patents, is followed with hyperinflammatory phase and cytokine storm where the most fatalities occur [2, 4]. If the viral and hyperinflammatory phases of the disease were not addressed early and properly, the patient might progress to ARDS

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which is almost fatal [2,6]. Hence, antiviral, anti-inflammatory, and immunomodulatory medications are necessary to stop the vicious progression of COVID-19 from mild-moderate to severe and to stop the clinical deterioration of the already severe patients.

Accordingly, Ivermectin and Doxycycline were used in this study because both drugs have shown antiviral and immunomodulatory activities [8-15]. Moreover, Doxycycline is a broad spectrum antibiotic which tackles the problem of secondary bacterial infection in COVID-19 patients [16-20]. Both drugs are FDA approved and have a high historical safety record [8,10,16, 19]; moreover, no interaction is known between Ivermectin and Doxycycline or between Ivermectin-Doxycycline and any of the medications given in the standard care. By contrary, for example, Azithromycin with Hydroxychloroquine are known to interact adversely for prolonging QT interval of cardiogram which might lead to serious complications [23].

Using FDA approved and safe antiviral and immunomodulatory medications for COVID-19 is scientifically justified for untreatable disease like COVID-19. It has been found that most of COVID-19 patients who progress to severe/critical disease have high viral load of SARS-CoV-2 and over-reacting immune response [24]. Therefore, reducing the viral load and dampening the immune response and inflammatory cytokines are necessary to save patients' lives.

The findings of the current trial showed that Ivermectin-Doxycycline reduced the mean time to recovery from 17.9 to 10.61 days in the recruited COVID-19 patients. Alike, for mild moderate patients, Ivermectin-Doxycycline reduced mean time to recovery from 13.66 to just 6.34 days with reduction in time up to 7.32 days. Nevertheless, Ivermectin-Doxycycline reduced the mean time to recovery in severe patients only 4 days, from 24 to 20 days. Based on these findings, Ivermectin and Doxycycline protocol proves to be effective in speeding up recovery in both mild-moderate outpatients and severe inpatients. This has a tremendous effect on lowering the burden of the disease, minimizing chances of developing immune deregulation, and freeing as quickly as possible hospital beds to other patients. This adds further evidence that Ivermectin-Doxycycline could exert both antiviral and immunomodulatory actives. Several observational studies showed that Ivermectin

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with/without Doxycycline shortens the time needed to recover COVID-19 patients and Ivermectin is beneficial for mild-moderate as well as severe patients [8-15].

In the current study, Ivermectin-Doxycycline arm lowered the rate of progression of the severe patients from 31.81% to as low as 9%. More interestingly Ivermectin-Doxycycline abolished death in severe patients, 0% mortality rate, compared to control arm, 27.27%. It is noteworthy to mention that the non-progression of the disease and the zero mortality in mild-moderate patients in both arms of the study might be attributed to the early diagnosis and therapy; moreover, the current standard care has become more effective than that used in the early months of the pandemic. However, larger study population is required to trace differences in the disease progression or the mortality rate of mild-moderate patients of COVID-19 taking Ivermectin-Doxycycline compared to patients taking standard care.

Accordingly, the present clinical trial reveals that Ivermectin-Doxycycline might stop disease progression and reduce death rate in severe patients of COVID-19. An observational preprint study conducted in Florida showed that Ivermectin cuts mortality rate of severe COVID-19 patients from 80.7% to 38.8% [25]. Interestingly, both Ivermectin and Doxycycline concentrations in the tissue of the lung have been estimated 2 times more than that in plasma [26, 27]. Therefore, their antiviral and anti-inflammatory effect on pulmonary tissues is expected to be prominent. These findings provide evidence that Ivermectin might be a potent immunomodulatory in addition to being antiviral agent. Nevertheless, these observational findings still need confirmation by a large randomized controlled study.

In fact, the observed benefits of Ivermectin-Doxycycline on the enrolled COVID-19 patients cannot be separated from the effect of the standard care, including Vitamin D and C, Zinc, and steroids, which was given concomitantly. Therefore, giving Ivermectin-Doxycycline along with Zinc, Vitamins D and C, and steroids at the viral and/or the hyperinflammatory phase of the disease seems clinically of benefit. This might shape the future most-fit combinational therapy of COVID-19 patients to minimize as could as possible the death rate and to decrease the duration and the progression of the disease.

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Short Communication

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Effectiveness of ivermectin-based multidrug therapy in severely hypoxic, ambulatory COVID-19 patients

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Aims: Ivermectin is a safe, inexpensive and effective early COVID-19 treatment validated in 20+ random, controlled trials. Having developed combination therapies for *Helicobacter pylori*, the authors present a highly effective COVID-19 therapeutic combination, stemming from clinical observations. **Patients & methods:** In 24 COVID-19 subjects refusing hospitalization with high-risk features, hypoxia and untreated moderate to severe symptoms averaging 9 days, the authors administered this novel combination of ivermectin, doxycycline, zinc and vitamins D and C. **Results & conclusions:** All subjects resolved symptoms (in 11 days on average), and oxygen saturation improved in 24 h (87.4% to 93.1%; $p = 0.001$). There were no hospitalizations or deaths, less than ($p < 0.002$ or 0.05, respectively) background-matched CDC database controls. Triple combination therapy is safe and effective even when used in outpatients with moderate to severe symptoms.

Clinical Trial Registration: NCT04482686 (ClinicalTrial.gov)

First draft submitted: 15 January 2022; Accepted for publication: 25 January 2022; Published online: 9 February 2022

Keywords: coronavirus • COVID • doxycycline • ivermectin • SARS-CoV-2 • zinc

There is currently a lack of effective treatments for early or ambulatory patients with COVID-19. Patients testing positive are sent home to isolate without specific treatment prescribed. However, there is growing evidence that certain repurposed drugs with good safety profiles, taken early, can significantly improve outcomes and even avoid or delay the need for immune-modulators, antiplatelet/antithrombotic therapy and the administration of oxygen [1].

Among the most extensively studied of such COVID-19 therapeutics is ivermectin (IVM), a drug that has been used safely in 3.7 billion doses worldwide since 1987 [2–4]. Recently, Dr Satoshi Omura, the 2015 Nobel Prize co-laureate for the discovery of IVM, and colleagues comprehensively reviewed studies to date on IVM activity against COVID-19, concluding that the evidence demonstrated such efficacy [4]. IVM used alone has been tested in more than 20 randomized, controlled trials (RCTs) for COVID-19 treatment, with statistically highly significant clinical benefits in almost all of these and an average of 62% reduction in risk of death [5]. Five such studies for IVM treatment of COVID-19 recently published in top-tier medical journals have all shown multiple clinical benefits for IVM versus controls, most of these with high statistical significance on the order of $p < 0.002$ [6–10]. At much greater than the standard single antiparasite dose of 200 μg/kg, IVM is well tolerated [11,12] and has been used in RCTs for COVID-19 treatment at cumulative doses of 1500 μg/kg [13] and 3000 μg/kg [14,15] over 4 or 5 days either without or with mild and transient adverse effects. Not surprisingly, IVM has become extensively used in the prevention and early disease management of COVID-19, particularly in non-Western countries.

Despite this strong evidence of clinical benefit in COVID-19 for IVM therapy, variation in therapeutic regimens, especially with respect to the addition of a broad-spectrum antibiotic and zinc, has led to confusion as to how best to manage acute infections. Indeed, the most impressive of the early ambulatory multidrug therapies, claiming

FOI 26-3132 - Document 5

87% and 75% reductions in hospitalization and deaths, respectively, both with a p-value of 0.001, in 869 high-risk subjects, left optimal management strategy unclear due to a mixed use of IVM and hydroxychloroquine (HCQ) [15]. There is an immediate need for an effective, safe and practically available combination therapy formulation based on the best available data.

At a cellular level, IVM modulates communication between the cytoplasm and nucleus, creating a hostile environment for assemblage of the virus while reducing cytokine-mediated inflammation. In addition, IVM inhibits pathology following infection with the COVID-19 virus, by specifically blocking the binding of the virus 'spike' protein to the ACE2 receptor. Finally, IVM has been associated with favorable changes in cellular innate immunity [16].

The authors' group has been systematically developing antiviral drug combinations for COVID-19 and found IVM to be particularly effective as a co-therapy for use early in COVID-19 to shorten the time to symptom resolution and to prevent hospitalization. IVM used alone can at times be only partially effective but not curative [1,6,17]. Thus, the authors chose a combination of safe and widely available medications, approved for other indications and without drug–drug interactions or QT prolongation and one that inhibits intracellular virus replication and possesses some anti-inflammatory properties.

The use of combination therapy for intracellular bacterial infections is not new and has been used successfully to treat tuberculosis, *Helicobacter pylori* (*H. pylori*) infections, leprosy and intracellular viral infections such as hepatitis B and C, in which a single component of the combination therapies is rarely curative. In some viral infections such as HIV, even combined multiple antiviral drugs cannot completely cure but suppress the viral load perpetually [18]. IVM is best known for its broad-spectrum efficacy for parasite infections, its high cure rate and its limited drug resistance when used in combination [19]. Although useful, IVM alone is not the 'magic bullet'. Combinations can help lower individual doses and reduce side effects. To cover all age group requirements, the authors combined IVM with doxycycline and zinc as active components and with vitamins D and C as replacement 'excipients' given to supplement common clinical deficiencies in the aged.

This study reports the use of the above combination therapy in consecutive, ambulatory, complex and at times profoundly hypoxic patients who insisted on avoiding hospitalization and whose oxygen saturation (SpO_2) was as low as 73%. Participants were treated by an experienced clinical trials team running a trial called Combination Therapy to Treat COVID-19 Infection.

Methods

Subjects

Subjects were identified from patients referred by physicians or word-of-mouth in Los Angeles, Ventura County, CA, and other states in the USA. These patients were referred to participate in clinical trials under clinicaltrial.gov ID NCT04482686 (which is a double-blind RCT). However, some did not qualify for this trial, as their SpO_2 was less than 90%, and were deemed too sick to enter a placebo-controlled trial. All subjects refused hospitalizations for different reasons, including not wanting to die in the hospital. Given subjects were excluded from other studies and refused to go to the hospital, they were treated off-label via telemedicine, during August 2020 and February 2021. Subjects were given the opportunity to participate in this open-label trial with institutional review board (IRB) oversight once the diagnosis was made via swab reverse transcription quantitative real-time PCR (RT-qPCR) testing. Inclusion criteria were as follows: positive PCR for COVID-19, informed consent, age $\geq$18 years old and agreement to practice two highly effective methods of birth control if of childbearing potential. Exclusion criteria were allergies or drug interactions with the combination therapy components; listed comorbidities, including seizure risk; and pregnancy.

Treatment

Treatment began as soon as practical, within 72 h of patients presenting to Ventura Clinical Trials. All screened subjects met the inclusion criteria and were enrolled consecutively. Treatment was defined as 'IVM Combination Therapy' (ICT) and consisted of 10 days of oral doxycycline (100 mg twice a day), IVM (12 mg on day 1, day 4 and day 8), zinc (25 mg twice a day), vitamin D3 (1500 IU twice a day) and vitamin C (1500 mg twice a day). ICT was given daily for 10 days only.

Two patients (#10 and #23) received an initial treatment on day 1 of 36 mg IVM (rather than 12 mg) due to particularly low SpO_2 or expected clinical need.

Monitoring

Subjects self-recorded symptoms in their daily logs (Supplementary Figure 1) for the first 10 days. Electrocardiograms (EKGs), blood pressure, temperature (reported in °F) and SpO_2 were collected via provided medical equipment at home. On days 1, 5, 10 and 30, SARS-CoV-2 testing swabs were self-collected by subjects and sent to pathology for testing. Pregnancy tests were performed as appropriate.

End points

End points were time from presentation to negative SARS-CoV-2 PCR, time from presentation to symptom resolution, progression to hospitalization and patient survival.

Externally controlled trial arm

Given the challenges for COVID-19 of enrolling high-risk, severely hypoxic patients in an open-label trial with an untreated control arm, the treated group arm survival was compared with the control group survival rate in the general population. This externally controlled trial (ECT), also known as a synthetic control arm, was calculated from the public CDC database of COVID-19 subjects [20]. Available information includes age range, presence of any chronic condition (COVID-19 vulnerability or otherwise, conditions not specified), date of infection and whether the COVID-19 diagnosis was laboratory confirmed. The authors used information from all subjects who met the following criteria: age 50+ years old; laboratory-confirmed COVID-19 diagnosis; death/survival, race and sex status available and known; infection prior to March 2021; and any comorbidities. This synthetic control arm data selection was carried out after the authors' data were obtained, to more closely match their subjects, all of whom had some comorbidity and the majority were over 50 years of age. The CDC database was analyzed using CSViewer version 1.3 (EasyMorph, Inc., ON, Canada, http://easymorph.com).

Covidex calculations & statistics

Covidex and Covidex-F are ambulatory SARS-CoV-2 infection disease severity measures that the authors developed and validated in this study. They are weighted particularly to emphasize SpO_2, and Covidex-F includes a variable for body temperature.

Covidex score = 1 point (pt) (if history of sleep apnea) + 1 pt (if history of chronic obstructive pulmonary disease [COPD]) + 1 pt (if history of cardiovascular disease) + 1 pt (if history of asthma) + 1 pt (if history of prior clots, ischemia or stroke) + 1 pt (if obese, that is, BMI between $\geq$30 kg/m^2 and <40 kg/m^2) + 2 pts (if severely obese, that is, BMI $\geq$40) + 1 pt (if age $\geq$60 years old) + (95-[SpO_2 as a percentage]) pts. For instance, a hypothetical patient with a history of asthma and morbid obesity with an SpO_2 prior to treatment of 85% would have a Covidex score of 1 (for asthma) + 2 (for obesity) + 10 (for SpO_2 of 85%) = 13 pts. Covidex-F score = Covidex score + 1 pt (if temperature on presentation between 99.5°F and 100.4°F) + 2 pts (if temperature on presentation between 100.4°F and 103.5°F) + 3 pts (if temperature on presentation $\geq$103.5°F).

Best-fit lines were made to assess the correlation between Covidex scores and time from treatment to symptom resolution. Regression was carried out in Prism version 8 (GraphPad Prism software for Windows, CA, USA, www.graphpad.com) using least square regression without weighting or special handling of outliers. All graphs were prepared by and statistical analysis was done using GraphPad Prism version 8, and the error bars are indicative of the standard error of the mean (SEM).

Results

Table 1 lists all subjects in the study, two of whom did not consent to ICT treatment (subjects #10 and #26, n = 26; n = 24 consenting to treatment), and their associated race, gender, symptoms and fever and other clinical notes. All subjects had COVID-19-related symptoms on presentation, and the symptom range was broad, with several showing shortness of breath (SOB). The vast majority of subjects, 21 of 24 (87.50%), had fever on presentation with a mean temperature for all 24 subjects of 101.2 ± 0.32°F. Specifically, 1/24 (4.17%) had low-grade fever (99.5–100.4°F), 18/24 (75.00%) had medium-grade fever (100.5–103.4°F) and 2/24 (8.33%) had high-grade fever ($\geq$103.4°F).

Given the dates of the study, it is very unlikely any subjects had the Delta or Omicron variant of COVID-19. Six subjects had their strains identified (data not shown) and were found not to have these variants.

Table 2 summarizes the demographics and past medical history (PMH) of subjects who consented to treatment (total n = 24; two additional subjects who declined treatment are excluded). Notably, patients were older (a known

FOI 26-3132 - Document 5

Table 1. Listing of subjects and COVID-associated symptoms on presentation and other characteristics.

ID	Age (years)	Race	Gender	Symptoms	Temp (°F)	O_2% sat.	O_2% sat. post 24 h	Rx start date	Symptom resolution date	PCR pos. date	PCR neg. date
1	66	Caucasian	M	Runny nose, sore throat, dizzy, low energy	99.3	90	94	12/14/20	12/21/20	11/6/20	1/17/21
2	62	Caucasian	M	SOB, chest congestion, productive cough, nausea, vomiting	105	77	87	12/8/20	12/18/20	11/30/20	12/18/20
3	75	Caucasian	M	Low energy	101	88	96	10/26/20	11/1/20	10/15/20	10/30/20
4	66	Caucasian	F	Loss of appetite, cough, chills, SOB	101	97	96	10/26/20	10/29/20	10/15/20	10/30/20
5	66	Caucasian	F	Vomiting, weak, body aches, anosmia	101	89	95	12/18/20	12/22/20	12/18/20	NA
6	43	Caucasian	F	PE, headache, body ache, cough	101	88	94	1/26/21	2/12/21	1/26/21	2/9/21
7	62	Caucasian	M	Productive cough, headache	102	86.5	91	11/24/20	12/8/20	11/13/20	12/8/20
8	57	Caucasian	M	Cough, nasal congestion, SOB, body aches	102	88	96	10/27/20	11/10/20	10/26/20	11/15/20
9	94	Hispanic	F	Low energy, SOB, confusion, loss of appetite, shaking	102	88	94	1/10/21	1/20/21	12/22/20	NA
10	66	Hispanic	M	Cough, SOB, respiratory failure	100.6	72	87	Declined	Death	12/22/20	NA
11	63	Hispanic	F	Cough, SOB	102	90	96	1/10/21	1/20/21	12/22/20	NA
12	47	Hispanic	M	SOB	104	84	91	12/19/20	12/25/20	12/16/20	NA
13	69	Caucasian	F	Cough, congestion, rash	102	88	91	11/17/20	12/3/20	11/13/20	NA
14	69	Caucasian	M	Post-nasal drip, cough, sinus pain	98	88	91	11/17/20	12/3/20	11/13/20	NA
15	71	Hispanic	M	Low energy, productive cough, anosmia	101	88	NA	12/17/20	1/5/21	12/13/20	NA
16	67	Hispanic	F	Dry cough, body aches, low energy, anosmia	100	88	NA	12/17/20	1/5/21	12/13/20	NA
17	46	Caucasian	F	Diarrhea, rash, renal pain	102	87	94	8/8/20	8/19/20	7/2/20	NA
18	86	Caucasian	M	Cough, fever, low energy	102	88	95	1/9/21	1/19/21	1/8/21	1/19/21
19	59	Caucasian	F	Stomach pain, diarrhea, cough, rash	102	90	95	9/16/20	9/25/20	8/19/20	NA
20	54	Muslim	M	Cough, fever, loss of appetite, chills	101.2	88	NA	10/16/20	10/28/20	10/15/20	10/28/20
21	92	Caucasian	M	Low energy	102	85	91	2/5/21	2/11/21	2/2/21	2/15/21
22	63	Hispanic	M	Cough, low energy, loss of appetite	101.3	90	96	2/2/21	2/12/21	2/2/21	NA
23	57	Hispanic	M	Cough, SOB	98	73	90	1/6/21	2/8/21	12/30/20	1/24/21
24	46	Hispanic	F	Chest pain, SOB	98.6	90	NA	2/18/21	2/24/21	2/17/21	NA
25	87	Hispanic	M	Severe SOB, low energy, trouble walking	101.6	90	95	2/27/21	3/5/21	2/17/21	NA
26	86	Caucasian	M	SOB	102	88	NA	Declined	Death	10/6/20	NA

ID: Identification number; NA: Not available; neg.: Negative; O_2% sat.: Oxygen saturation just before time of treatment initiation; O_2% sat. post 24 h: Oxygen saturation 24 h after treatment initiation; PE: Pulmonary embolism; pos: Positive; Rx start date: Day 1 of IVM Combination Therapy administration; SOB: Shortness of breath; Temp.: Temperature.

FOI 26-3132 - Document 5

Table 2. Demographics and clinical characteristics of subjects.

Age and demographics of subjects		
Age (mean, SEM, range)	66, 2.75, 43–94	
Male, n (%)	15 (62.5)	
Female, n (%)	9 (37.5)	
Race, n (%)		
– Caucasian	14 (58)	
– Hispanic Mexican	7 (27)	
– South American	2 (8)	
– Other	1 (4)	
Prevalence of COVID susceptible comorbidities		
Comorbidity	**Subjects (n)**	**Subjects (%)**
Type 2 diabetes mellitus	6	25.00
Heart or cardiovascular	5	20.83
COPD	3	12.50
Obesity (BMI 30–40)	3	12.50
Severe obesity (BMI ≥40)	2	8.33
Chronic kidney disease	1	4.17
Immunocompromised state	1	4.17
Concurrent comorbidities in subjects (n)		
Comorbidities (n)	**Subjects (n)**	**Subjects (%)**
0	13	54.17
1	6	25.00
2	3	12.50
3	2	8.33

COPD: Chronic obstructive pulmonary disease; SEM: Standard error of the mean.

COVID-19 vulnerability), with a mean age of 66 ± 2.75 years old and a range of 43–94 years (Table 2A). The population of the 24 subjects consenting to treatment (not subjects #10 and #26, Table 1) was 63% males. Death of untreated subjects #10 and #26 excluded downstream analysis in the other figures.

Table 2B lists the number of patients who had comorbidities associated with COVID-19 vulnerability, based on CDC guidelines [21]. These comorbidities are chronic kidney disease, COPD, Down syndrome, cardiovascular disease, immunocompromised state (including HIV), obesity (BMI of 30 kg/m^2 or higher but <40 kg/m^2), severe obesity (BMI ≥40 kg/m^2), pregnancy, sickle cell disease, smoking and Type 2 diabetes. Of note, no subjects had cancer, Down syndrome or sickle cell disease and none were pregnant or were smokers.

Many subjects had multiple comorbidities associated with COVID-19 vulnerability, as outlined in Table 2B. In total, 11/24 (45.83%) subjects had COVID-19-vulnerable comorbidities, of which three (12.50%) had two separate comorbidities and two (8.33%) had three comorbidities.

A minority of subjects (n = 7) had other COVID-19 treatment(s) prior to and/or during ICT administration, namely remdesivir (n = 1 subject); involvement in a placebo-controlled trial of hydroxychloroquine, azithromycin, vitamin D, zinc pack (HAZDpaC) (n = 4 subjects, trial clinicaltrial.gov NCT04334512; may have been given treatment or placebo); and hydroxychloroquine (HCQ) (n = 3 subjects).

Figure 1 demonstrates that all subjects recovered from COVID-19, typically within 1 to 2 weeks. Figure 1A shows various durations for each subject and average values (one outlier excluded). Time from onset of symptoms to treatment initiation is shown in column 1 and averaged 9.2 ± 2.1 days. The time from start of treatment to symptom resolution was 11.6 ± 1.4 days. Time from first positive to first negative PCR was 16.9 ± 1.6 days and was less than 3 weeks. The time from start of treatment to first negative PCR was 11.5 ± 1.6 days and was also less than 3 weeks.

Figure 1B shows that 100% of subjects survived COVID-19, without the need for hospitalization or ventilator use. As noted in Table 2, many of these subjects were older and with comorbidities. When compared with the CDC synthetic control arm (see methods and as follows), this was a significant increase in survival rate ($p = 0.044$) and decrease in hospitalization rate ($p = 0.0011$), evaluated via χ^2 test. Of note, the patients in the CDC database likely

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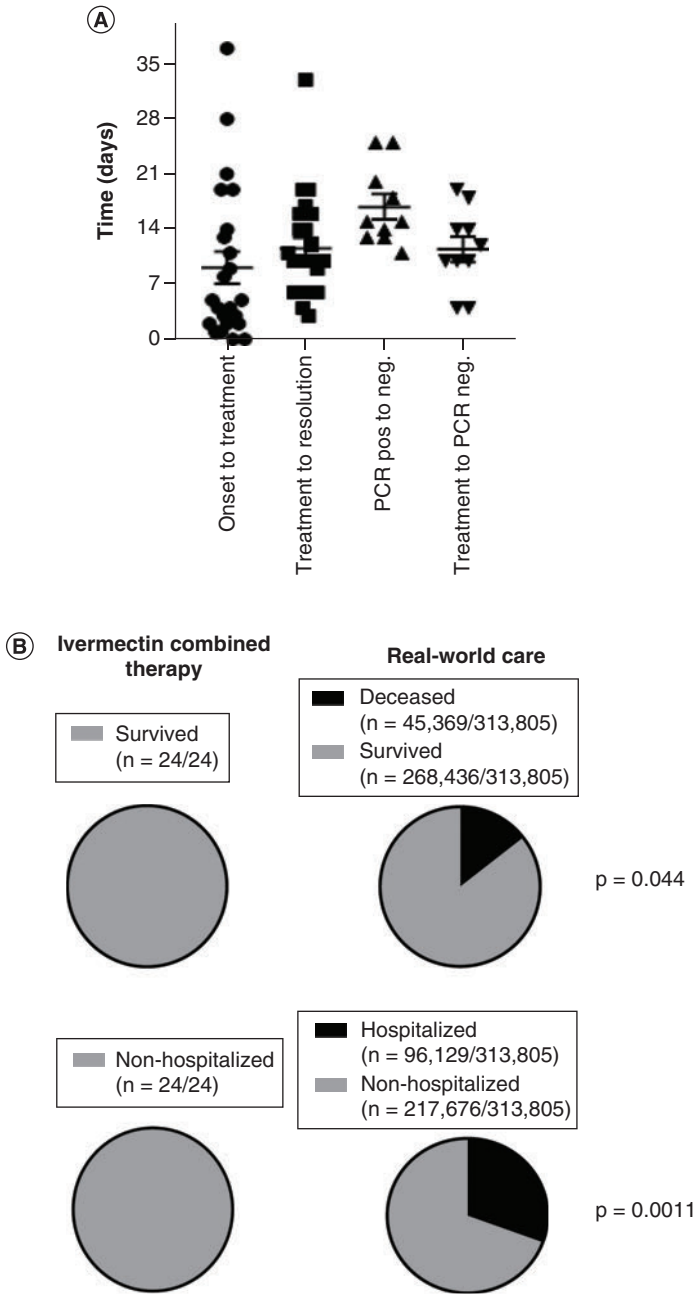


Figure 1. Complete recovery was seen in all patients within 1 to 3 weeks. (A) Time in days to various stages of symptom onset and resolution. Nearly all subjects resolved symptoms and became PCR negative in 3 weeks.
Col 1: Symptom onset to start of treatment (n = 23; mean: 9.17 ± 2.05).
Col 2: Start of treatment to resolution of symptoms (n = 23; mean: 11.61 ± 1.38).
Col 3: First PCR positive to first PCR negative (n = 10; mean: 16.90 ± 1.58).
Col 4: Start of treatment to first PCR negative (n = 10; mean: 11.50 ± 1.60).
(B) Top, 100% survival rate was seen in patients, which is significantly higher (p = 0.044 via Chi-Square, χ^2, test) than synthetic control from CDC database of equivalent or less COVID-vulnerable subjects. Bottom, no (0%) patients required hospitalization, which is significantly less (p = 0.0011 via χ^2 test) than synthetic control from database.

received treatment of an unknown nature. Thus, the survival rate of this synthetic control reflects the "typical" survival rate in the USA, which is significantly less than the 100% survival rate observed on ICT.

The 100% survival rate on ICT was compared with survival rates from the CDC database of COVID-19 subjects; 313,805 control subjects were obtained, based on the qualification criteria described in the methods section. These criteria focused on older subjects (50+ years of age), similar to the authors' population, who also had underlying conditions. One should note that the underlying condition criteria information available in this database refers to chronic conditions of any type, whether or not the condition induces COVID-19 vulnerability. With this definition, 100% of control subjects and ICT-treated subjects had underlying conditions of any type.

The critical finding in Figure 2 (see also Table 2) showed that 23/24 patients were hypoxic with SpO_2 < 90%. Some subjects consenting to treatment had SpO_2 as low as 73%, 77%, 84% and 85% on presentation. Figure 2A shows that the SpO_2 of subjects was significantly less than 95%, the defined point of cure (95% CI of mean SpO_2: 85.5% to 89.4%; mean: 87.4% ± 0.93). Subjects' SpO_2 increased within 24 h of treatment. Their mean SpO_2

FOI 26-3132 - Document 5

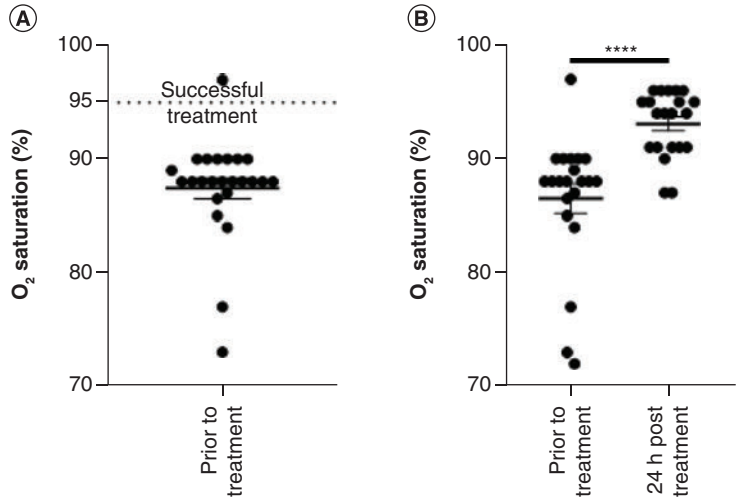


Figure 2. All subjects reached successful treatment (SpO2 above 95%) and experienced significant increase in SpO2 within 24 h. (A) O_2 saturation prior to treatment was significantly ($p < 0.05$) less than 95%, the defined successful treatment reached by all subjects. **(B)** O_2 saturation significantly increased in subjects 24 h after treatment (paired *t*-test, $p < 0.001$, only subjects with data before and after treatment included). O_2 saturation continued to rise until the defined cure of greater than 95% O_2 saturation was reached.

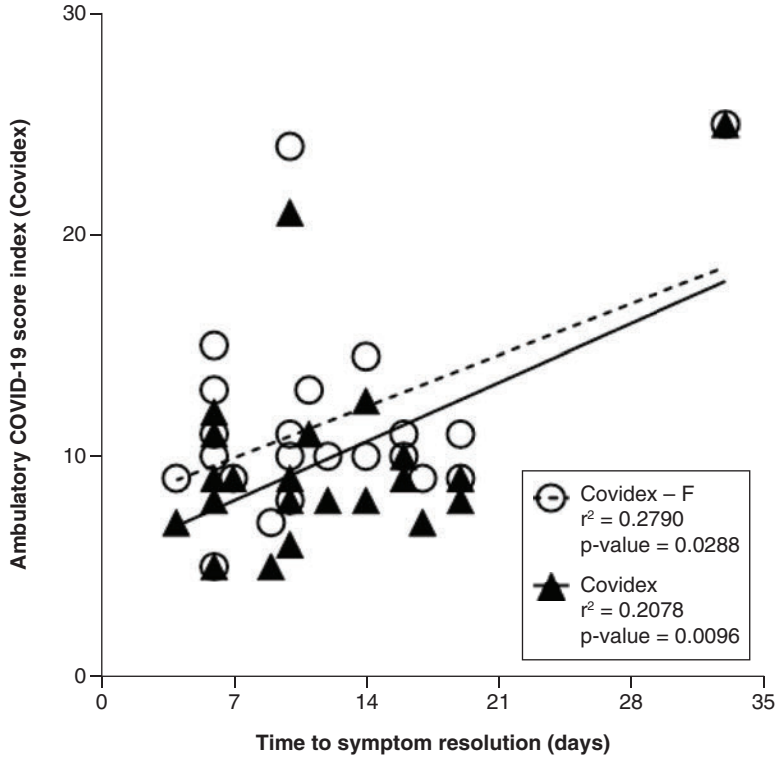


Figure 3. Ambulatory COVID scores, Covidex and Covidex-F (see methods for definition; Covidex-F includes fever measure) versus time from start of treatment to symptom resolution. There was a significant relation between either Covidex score (Covidex: p = 0.0288; Covidex-F: p = 0.0096) and treatment resolution time.

before treatment (for subjects with data before and after 24 h) was 86.5% ± 1.3, and after 24 h of treatment 93.1% ± 0.63, a highly significant and rapid increase ($p < 0.001$). SpO_2 then continued to rise. Treatment continued for 10 days reaching the point of successful treatment or cure, with $SpO_2 > 95\%$. Successful treatment was reached for all subjects. That is, there was a 100% restoration rate in terms of SpO_2. No patient who accepted treatment required hospitalization.

Two subjects (#10 and #26) declined treatment. These subjects did not recover SpO_2 and died from COVID-19 infection. An adverse drug event of dizziness was reported by one treatment subject who nevertheless continued with treatment successfully otherwise.

Figure 3 shows the validation of the Covidex and Covidex-F scores the authors developed, defined in the methods. These scores provide an index of COVID-19 predicted severity, based on PMH, O_2 just before treatment and fever grade on presentation. The mean Covidex score was 10.34 ± 1.08 and the mean Covidex-F score was 11.63 ± 1.13, with 87% and 85% of the score points, respectively, coming from the contribution of the SpO_2

10.2217/fmb-2022-0014

FOI 26-3132 - Document 5

term. In other words, approximately 80–90% of this score is weighted toward SpO_2. It should also be noted that the PMH aspects that contribute to this score emphasize respiratory, cardiovascular and obesity histories and differ from CDC-defined COVID-19 vulnerabilities.

Covidex and Covidex-F are both plotted versus time from treatment to symptom resolution. Both show statistically significant correlations (Covidex: p = 0.0096, r^2 = 0.2078; Covidex-F: p = 0.0288, r^2 = 0.2790), indicating that either Covidex or Covidex-F is associated with and may predict time to symptom resolution.

Discussion

The authors report for the first time a highly effective outpatient ICT, which prevented hospitalization and led to 100% survival and cure in unselected ambulatory 'moderately to severely' ill COVID-19 patients with hypoxia. Given the authors' experience developing combination therapies for *H. pylori*, they clinically explored (without formal comparative studies) several different IVM-based combinations on ambulatory COVID-19 patients, searching for a cure, and found the above combination to be very effective as a therapy for COVID-19. Some patients needed a personalized medicine approach, using HCQ or other components, much the way *H. pylori* resistant to triple therapy sometimes requires quadruple therapy. The authors acknowledge that they did not have the capacity in this study to test every permutation of ingredients with a scientific trial, yet such studies can be useful for future refinement. Applying IVM via weight-based dosing has been suggested [22], but more importantly, appropriate doses may be needed to cover various COVID-19 strains.

Hypoxia is a demonstrated predictor of COVID-19 mortality. For example, several of these patients had profound hypoxia, measured by oximetry at 73%, 77%, 84% and 85% on presentation. Despite a symptom to treatment delay of 9.2 days, this treatment brought rapid improvement – beginning in some within 12 h with a mean SpO_2 rising from 86.5 to 93.1 in the first 24 h. There was a parallel improvement in the symptoms, including loss of cough, fever and tiredness. Also, the time from the start of treatment to the first negative PCR averaged 11.5 $\pm$ 1.6 days. Generally, such ill patients would have been admitted to the hospital, yet all those treated with ICT avoided hospitalization and none died.

Turning to the ECT 'synthetic control arm', it is clear that ICT was statistically superior to the control arm, even though a small patient group was reported. The very low number of adverse effects from reported studies and this treatment group support the use of ICT if clinical symptoms and risk factors for COVID-19 progression are present, even in cases with PCR pending results. ECT arms are now increasingly used, especially where the control arm or 'standard-of-care' arm may have a fatal outcome [23].

The rationale for exploring a combination antiviral therapy approach is based on our growing understanding that intracellular infections – bacterial or viral – generally cannot be cured using a single drug. Multiple drugs address numerous mechanisms of viral replication and can cure multiple strains. Even therapies using two drugs, such as two antibiotics for *H. pylori*, have resulted in resistance. Multiple-drug therapies, especially using three or more, are expected to be more effective at reducing viral load, thereby limiting resistance and variant development [24], as reviewed for HIV [18]. Use of IVM alone, on the other hand, has already led to resistance when treating scabies, nematodes, strongyloidiasis, microfilaridermias and *Onchocerca volvulus* [17,25–28]. All these diseases are based on eukaryotic organism infection, not viral, and are thus far less prone to mutations and consequently resistance. Since IVM resistance has occurred on occasion for these diseases and since COVID-19 has become a pandemic, resistance needs to be proactively prevented for COVID-19 and its future variants. Given the current state of the pandemic, we cannot permit IVM resistance to develop clinically, and be experimentally demonstrated, before it is addressed. Thus, IVM in COVID-19 should not be used as a monotherapy, only in combination therapies, especially so with growing reports of mutant strains resulting in vaccine breakthrough infections. Combination therapy could allow for more rapid cures, resistance prevention and overcoming of mutant strain emergence – 'no replication, no mutation'.

Regarding strategies in the development of combination therapies, intracellular coronavirus replication requires several active drugs to inhibit viral replication. IVM, doxycycline and zinc all individually inhibit coronavirus replication and, although there are other candidates, the authors have proposed the above combination based on its efficacy, component safety profiles, inexpensive nature and lack of drug–drug interaction. The combination of IVM and doxycycline has been shown to be somewhat effective for COVID-19 [7], potentially (statistical trend) more effective than the HCQ and azithromycin combination [29], and mechanistically theorized as synergistic [30], even though doxycycline alone is not considered effective [31]. Further, given that zinc plays a key role in antiviral activity [32,33], it would combine well with the ionophores (IVM and doxycycline) to increase its intracellular

concentration and expedite viral clearance [34]. Also, the combination of zinc with IVM and doxycycline has no reported drug–drug interactions [35]. Additionally, each of these drugs has a low adverse side-effect profile and no QT prolongation as reported with azithromycin [22,36–38].

Overall, based on the current literature, a 10-day combination therapy of IVM, doxycycline and zinc will not only improve symptoms [6,7] but also accelerate recovery from COVID-19. The authors have chosen a safe IVM dosage, approved for parasites, of 36 mg over 10 days, and this dose has been shown to be both effective and safe in COVID-19 treatments [39]. The staggered IVM dosage over 10 days is proposed based on the half-life clearance of the drug in plasma (up to 66 h) [40]. The proposed duration would allow constant availability of adequate plasma level IVM to facilitate zinc entry into the cells. Hence, the above rationale explains why some publications have already shown that IVM alone is not adequate to cure COVID-19, while a multidrug regimen is likely to be more efficacious [41].

This study enrolled consecutive subjects (i.e., as subjects presented, they were enrolled and none were turned down) in the study and did not bias subject selection from different time points. Many of the enrolled subjects were profoundly ill with subjective assessments that may have resulted in hospital admission and/or intubation, yet ICT activity appears to have rapidly restored SpO_2 and reversed other symptoms, which could not be explained simply by the developing immunity. The authors acknowledge that a major limitation of the study is the small sample size (n = 24 consenting to treatment) in this preliminary study, which may limit its generalizability. These subjects consisted of the first set to present to Ventura Clinical Trials, and more data are being collected. Of note, many such severely ill patients may not opt for the unknown of a less established clinical trial for a serious case of COVID-19. The authors' future studies will expand the sample size, test the effect of other IVM dosages and explore the mechanistic contribution of the microbiome to IVM's effectiveness in COVID-19.

While a concomitantly enrolled control arm would be ideal for a true RCT, this is not feasible with severe COVID-19. Given the potentially fatal outcomes, Lawrie *et al.* [42] said, "*Placebo-controlled trials of ivermectin treatment among people with COVID-19 infection are no longer ethical and active placebo-controlled trials should be closed,*" the effects of which observed in the two subjects who declined treatment and did not survive. Hence, this study has made use of the ECT or 'synthetic' control arm [23], which has enabled the authors to make matched age and comorbidity comparisons. Institutional review boards should now include a provision to allow for synthetic arm studies and reject COVID-19 trials that utilize a control arm as published by Lawrie.

Conclusion

This study builds on extensive literature to provide practical, inexpensive, safe, readily available and highly effective IVM triple therapy aiming to prevent resistance and one that can confidently be used as a routine treatment for outpatient COVID-19.

Summary points

- The authors observed the effectiveness of a novel combination of ivermectin, doxycycline, zinc and vitamins D and C in 24 COVID-positive subjects with high-risk features/comorbidities.
- The majority of the subjects were hypoxic (23 out of 24; oxygen saturation [SpO_2] <90%), with 4 below SpO_2 <85% on presentation. Many subjects had related comorbidities (n = 11; 45.8%). The median age of all subjects was 66 years old.
- Intervention consisted of 10 days of doxycycline (100 mg twice a day), ivermectin (12 mg on day 1, day 4 and day 8), zinc (25 mg twice a day), vitamin D3 (1500 IU twice a day) and vitamin C (1500 mg twice a day). All treatment was given orally and ceased at the end of 10 days.
- One hundred percent of patients accepting treatment survived without the need for hospitalization. All subjects recovered from hypoxic symptoms (SpO_2 >95%) within 10 days of treatment.
- The authors report an effective and inexpensive COVID therapy preventing hospitalization and death in all patients and rapid resolution of hypoxia in these ambulatory 'moderately to severely' ill patients.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fmb-2022-0014

FOI 26-3132 - Document 5

Author contributions

S Hazan contributed to and led all aspects of the study, including idea formulation, clinical treatment, study design, Covidex design, analysis and writing. S Dave carried out data analysis and Covidex design and validation, obtained control and contributed to writing. AW Gunaratne, RL Clancy and PA McCullough contributed to writing. S Dolai contributed to writing and study design. TJ Borody led all aspects of the study, including idea formulation, study design and writing.

Financial and competing interests disclosure

This study was funded by ProgenaBiome, LLC. S Hazan declares that she has pecuniary interest in Topelia Pty Ltd in Australia and Topelia Pty Ltd in the USA where development of COVID-19 preventative/treatment options are being pursued. She has also filed patents relevant to coronavirus treatments. She is the founder and owner of Microbiome Research Foundation, ProgenaBiome and Ventura Clinical Trials. TJ Borody declares that he has pecuniary interest in Topelia Pty Ltd in Australia and Topelia Therapeutics, Inc. in the USA developing COVID-19 preventative/treatment medications. He has also filed patents relevant to COVID-19 treatments. S Dave declares she has corporate affiliation to McKesson Specialty Health / Ontada and North End Advisory, LLC, and affiliation to Microbiome Research Foundation (a non-profit). S Dave is unaware of and not directly involved in COVID-19 treatment–relevant projects at McKesson, but they may exist. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed

S Dave provided medical writing assistance for this paper and was funded by ProgenaBiome and Microbiome Research Foundation.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board (IRB) approval and have followed principles of the Declaration of Helsinki for all human experimental investigations. All subjects were explained the study and provided written informed consent. This study was approved by E&I Review Services (https://www.eandireview.com/) as study #210006. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Biochemical and Biophysical Research Communications 538 (2021) 163–172

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The broad spectrum host-directed agent ivermectin as an antiviral for SARS-CoV-2 ?

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ARTICLE INFO

Article history:
Received 15 October 2020
Accepted 18 October 2020
Available online 21 October 2020

Keywords:
Ivermectin
Antiviral
SARS-CoV-2
COVID-19
Dengue virus
Zika virus

ABSTRACT

FDA approved for parasitic indications, the small molecule ivermectin has been the focus of growing attention in the last 8 years due to its potential as an antiviral. We first identified ivermectin in a high throughput compound library screen as an agent potently able to inhibit recognition of the nuclear localizing Human Immunodeficiency Virus-1 (HIV-1) integrase protein by the host importin (IMP) α/β1 heterodimer, and recently demonstrated its ability to bind directly to IMPα to cause conformational changes that prevent its function in nuclear import of key viral as well as host proteins. Cell culture experiments have shown robust antiviral action towards a whole range of viruses, including HIV-1, dengue, Zika and West Nile Virus, Venezuelan equine encephalitis virus, Chikungunya, pseudorabies virus, adenovirus, and SARS-CoV-2 (COVID-19). Close to 70 clinical trials are currently in progress worldwide for SARS-CoV-2. Although few of these studies have been completed, the results that are available, as well as those from observational/retrospective studies, indicate clinical benefit. Here we discuss the case for ivermectin as a host-directed broad-spectrum antiviral agent, including for SARS-CoV-2.

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1. Introduction

The work identifying ivermectin, a macrocyclic lactone 22,23-dihydroavermectin B produced by the bacterium *Streptomyces avermitilis*, and subsequently demonstrating its activity as a novel therapeutic against "infections caused by roundworm parasites" was recognized, along with the seminal work on the antimalarial artemisin, with the award of the 2015 Nobel Prize for Physiology or Medicine [1,2]. Soon after its discovery in 1975, ivermectin was approved as a treatment for parasitic infection indications in animals (1981), and subsequently for human use to treat onchocerciasis (river blindness) 6 years later. It has since been approved for treatment of a range of human nematode (roundworm) infestations that cause river blindness, filariasis, ascariasis and strongyloidiasis, as well as pediculosis and scabies, caused by ectoparasites, and also for rosacea [1,3]. Because of its importance in treating all of these indications, ivermectin is firmly on the WHO (World Health Organisation)'s List of Essential Medicines [6], with millions of doses prescribed worldwide every year.

Starting in 2012, ivermectin's antiviral properties have been progressively documented towards a number of RNA viruses, including human immunodeficiency virus (HIV)-1, influenza, flaviviruses such as dengue and Zika, and most notably, SARS-CoV-2 (COVID-19) [4,5,7–17], as well as DNA viruses such as pseudorabies, polyoma and adenoviruses [18–20]. Ivermectin's antiviral activity is based on its ability to bind to and inhibit the transport function of the host importin α (IMPα) protein [11,18,20]; IMPα is known to mediate nuclear import of various viral proteins and key host factors, although other actions of ivermectin have been proposed which may also contribute to its activity [eg. [12,21,22]]. In light of the possibility that ivermectin has potential to be a key weapon to help control the SARS-CoV-2 pandemic, this review briefly examines the weight of evidence for ivermectin's broad-spectrum antiviral activity through its action on host factor IMPα, [6,17].

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https://doi.org/10.1016/j.bbrc.2020.10.042
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FOI 26-3132 - Document 5

2. FDA-approved anti-parasitic ivermectin

Ivermectin has had an immense impact as a therapeutic to control various parasitic diseases [1–6], its antiparasitic mode of action believed to be through potentiating GABA-mediated neurotransmission, and by binding to invertebrate glutamate-gated Cl^- channels to effect parasite paralysis and death [23]. The selectivity of ivermectin stems from the fact that it does not readily penetrate the central nervous system (CNS) of mammals, where GABA functions as a key neurotransmitter [23]. Doses up to 2000 μg/kg can be well tolerated in patients with parasitic infections [23,24], with analysis of the first 11 years of mass global ivermectin administration indicating a very low incidence of serious adverse side effects [4,25], with no resistance in humans yet confirmed in over 25 years. Ivermectin is in fact usually administered as a single oral yearly dose (eg. 150 or 200 μg/kg, respectively) to treat onchocerciasis and strongyloidiasis, with filariasis similarly treated with a once yearly dose (300–400 μg/kg) in endemic areas, or alternatively bi-yearly (150–200 μg/kg) [26]. Clearly, ivermectin is a safe, efficacious antiparasitic likely to remain an integral part of the WHO List of Essential Medicines [6] long into the future [1,4].

3. Ivermectin as an IMPα targeting agent

Transport into and out of the nucleus is an integral part of normal eukaryotic cell function, as well as in the case of viral infection, since viruses commonly hijack the system in order to antagonize the cellular antiviral response that is driven to a large extent by nuclear factors such as transcription factors [14,27]. The key signal-dependent mediators of this transport are the members of the IMP superfamily of proteins, of which there are multiple α and β forms [14,27]. Nuclear transport mediated by the IMPα/β1 heterodimer is the best characterized pathway by which host proteins enter the nucleus through nuclear envelope-embedded nuclear pores; host proteins transported into the nucleus include members of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and signal transducers and activators of transcription (STATs) inducible transcription factor families that play key roles in the response to infection. Specific viral proteins [see [27,28]] also use this pathway (see Fig. 1). Briefly, IMPα within the IMPα/β1 heterodimer performs the adaptor role of signal-specific recognition of the nuclear import cargo, while IMPβ1 performs the main transport roles of binding to/translocation through the nuclear pores, and subsequent release of the nuclear import cargo within the nucleus upon interaction with another transport factor, the monomeric guanine nucleotide binding protein Ran (not shown in Fig. 1) [see 27]. Specific high affinity recognition by IMPα is critical to nuclear localization of various viral proteins such as dengue non-structural protein (NS) 5, as shown in mutagenic studies [32]; significantly, dengue virus with impaired NS5 interaction with IMPα is severely attenuated, underlining the critical importance of the NS5-IMPα interaction for dengue infection. The importance of this interaction to dengue infection is the basis for the fact that multiple distinct small molecules that disrupt IMPα recognition of dengue NS5 are able to limit dengue infection [eg. [7,8,11,31,33]]. Significantly in the case of ivermectin, this activity extends to a large number of different viruses (see below) [7–17], including SARS-CoV-2 [18].

We identified ivermectin in 2011 in a high throughput screen using bacterially expressed proteins and a 1200-compound library of small molecules for inhibitors of HIV-1 Integrase (IN) recognition by IMPα/β1 [34]; specific inhibitors targeting IMPα/β1 directly (such as ivermectin) and not IN (such as budesonide) were identified using a nested counterscreening strategy [34,35]. Of several compounds now confirmed to be active against IMPα/β1 and possessing antiviral activity as a consequence [7,14,31,36], ivermectin is the best characterized, shown to have broad-spectrum activities (summarized in Tables 1–3). It was initially shown to inhibit nuclear import not only of IN, but also of simian virus SV40 large tumour antigen (T-ag) and other IMPα/β1- (but not IMPβ1-) dependent cargoes, consistent with the idea that IMPα (not IN) is the direct target of ivermectin [34,35]. Subsequent work has confirmed this, with ivermectin's ability to inhibit the nuclear accumulation of various different host, including NF-kB p65 [37,38] and viral proteins demonstrated in transfected and infected cell systems (see Table 1) [14,34]. Ivermectin's ability to inhibit binding of IMPα to the viral proteins NS5 and T-ag has also been confirmed in a cellular context using the biomolecular fluorescence complementation technique [11] (Table 1).

Although targeting of IMPα by ivermectin was implicated by many years of research (see also below), direct binding was only recently formally demonstrated using a set of biophysical techniques, including thermostability, analytical ultracentrifugation, and circular dichroism (CD) [11]. Importantly, the CD/thermostability studies indicate that binding of ivermectin to IMPα induces structural changes which are likely the basis of ivermectin inhibition of IMPα binding to viral nuclear import cargoes. Strikingly, the structural change also appears to impair heterodimerisation of IMPα with IMPβ1 [11 - see however 20]; thus, ivermectin inhibits nuclear import not only by preventing signal recognition by IMPα, but also by ensuring that the IMPα/β1 complex essential to mediate subsequent transport through the nuclear pore is prevented from forming (see Fig. 1).

4. Ivermectin's broadspectrum antiviral activity

Consistent with the fact that many viruses are known to rely on IMPα/β1-dependent nuclear import of specific viral proteins for robust infection, including many viruses for which the lifecycle is entirely carried out in the cytoplasm [14,27,28], a body of *in vitro* studies have confirmed ivermectin to be active in limiting infection by a range of different RNA viruses [10,14], including HIV-1 [7], DENV (all 4 serotypes) and related flaviviruses [8,11,12], influenza, and alphaviruses such as Venezuelan equine encephalitis virus (VEEV) and Chikungunya [9,15,16] (see Table 2); it is also active against DNA viruses [18–20]. Exciting recent studies indicate it is a potent inhibitor of SARS-CoV-2 [17].

A striking aspect of this antiviral activity is that, where determined, the EC50 for viral inhibition as assessed by a range of different techniques is in the low uM range (see right column, Table 2), interestingly aligning perfectly with its activity in inhibiting recognition of viral nuclear import cargoes by IMPα (see Table 1). The clear implication is that the mechanism of inhibition of infectious virus production in the case of all of the viruses listed in Table 2 is largely through targeting IMPα to prevent its role in nuclear import, and of viral proteins in particular (see Fig. 1). Significantly, two other small molecules (GW5074 and gossypol) that appear to target IMPα in a very similar way to prevent its nuclear import function [31 and unpublished] have comparable antiviral properties [13,31,36], consistent with the idea that the host protein IMPα is a key contributor to infection by a number of medically important viruses.

5. Ivermectin in the clinic as an antiviral ?

As in many other disciplines, one of the biggest challenges in antiviral research is to transition from laboratory experiments to preclinical/clinical studies, with the question of dosing in the case of ivermectin for viral infectious indications contentious [see 6,39,40]. It is important, firstly, to stress the obvious in this context:

FOI 26-3132 - Document 5

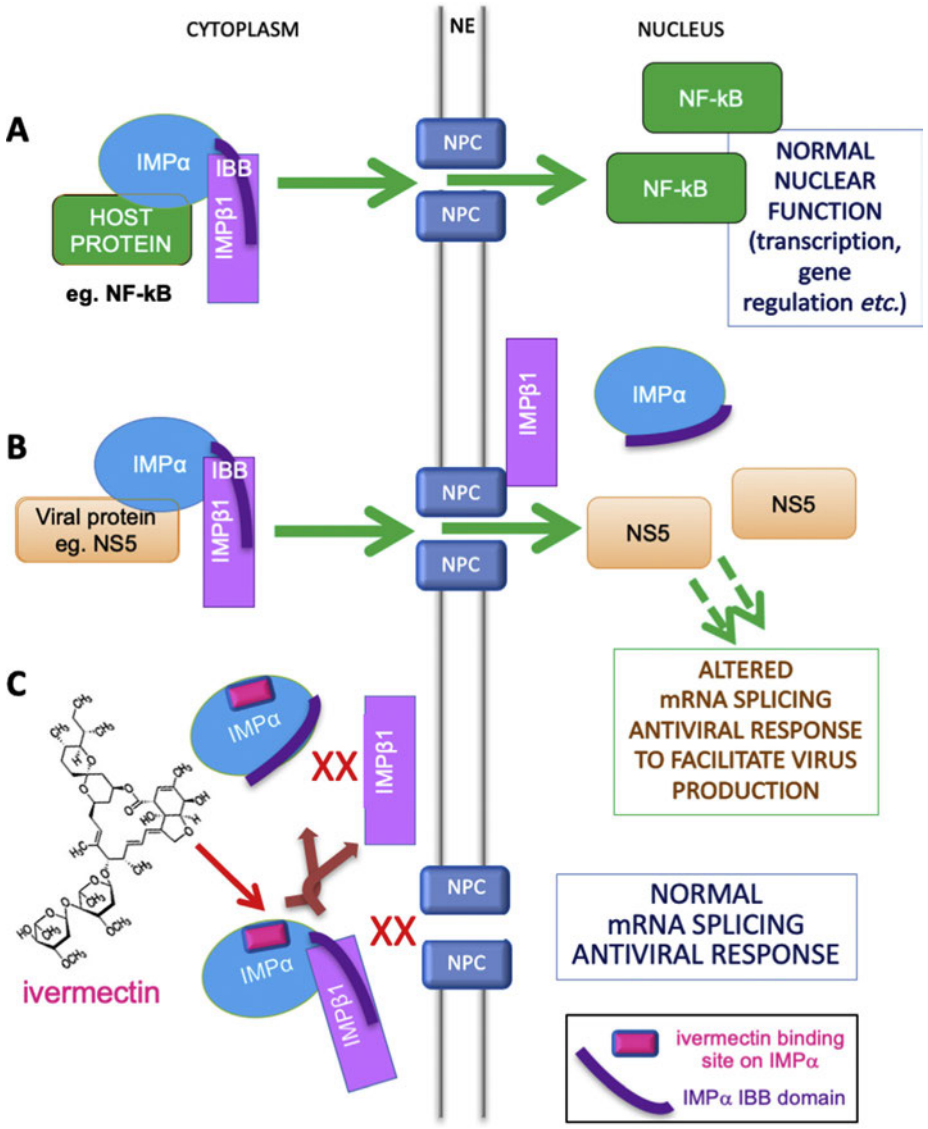


Fig. 1. Model of IMPα′s role in nuclear transport of host and viral proteins, and mechanism of inhibition by ivermectin. **A.** Host proteins such as members of the NF-κB transcription factor family localize in the nucleus through IMPα/β1, where the "IBB" (IMPβ-binding) region of IMPα (purple curved line) is bound by IMPβ1 to enable cargo recognition by IMPα within the heterodimer. Subsequently, IMPβ1 mediates transport of the trimeric complex into the nucleus through the nuclear envelope (NE)-embedded nuclear pore (NPC, nuclear pore complex). Release within the nucleus enables the transcription factor to carry out normal function in transcriptional regulation, including in the antiviral response. IMPα cannot mediate nuclear import, unless heterodimerised with IMPβ1. **B.** In viral infection, specific viral proteins (eg. NS5 in the case of DENV, ZIKV) able to interact with IMPα utilize the IMPα/β1 heterodimer to access the nucleus and antagonize the antiviral response [14,27,28]. This is critical for optimal virus production as shown by mutagenic and inhibitor studies. The SARS-CoV-2 proteins that may access the nucleus via IMPα/β1 in infected cells has not been examined, but in ORF6 (Open Reading Frame 6) protein from SARS-CoV1 has been shown to bind IMPα [29], and ORF4b from MERS-CoV (Middle Eastern Respiratory Syndrome Coronavirus) are both known to access the nucleus in NLS-dependent fashion [30]. **C.** The small molecule ivermectin (structure shown) binds to IMPα (binding site shown as red lozenge) both within the IMPα/β heterodimer to dissociate it, and to free IMPα to prevent it binding to IMPβ1, thereby preventing NS5 nuclear import [11], contributing to reduced virus production. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that the antiviral activities of ivermectin documented in Table 2 have been derived from laboratory experiments that largely involve high, generally non-physiological, multiplicities of infection, and cell monolayer cultures, often of cell lines such as Vero cells (African green monkey kidney cells impaired in interferon α/β production) that are not clinically relevant. Clearly, the results in Table 2 for low uM EC50 values reveal robust, dose-dependent antiviral activity in the particular model system used, but it would be naïve to believe that it is necessary to achieve uM concentrations of ivermectin in a patient for maximum clinical benefit.

In fact, a key consideration in clinical intervention using ivermectin is its host-directed (IMPα-directed) mechanism of action. Host-directed agents that impact cellular activities that are essential to healthy function must be tested with caution; although ivermectin has an established safety profile in humans [24,26], and is FDA-approved for a number of parasitic infections [1,3,5], it targets a host function that is unquestionably important in the antiviral response, and titration of a large proportion of the IMPα repertoire of a cell/tissue/organ is likely to lead to toxicity. With this in mind, where a host-directed agent can be a "game-changer" in treating viral infection may well be in the initial stages of infection or potentially even prophylactically by keeping the viral load low so that the body's immune system has an opportunity to mount a full antiviral response [11,17].

FOI 26-3132 - Document 5

Table 1
In vitro effects of ivermectin on IMPα.[a]

Effect on IMPα	Cargo Protein	
	Virus	Host
In vitro		
Inhibits direct binding of IMPα (ALPHASCREEN/AUC)	HIV IN (IC50 5 μM) {34] DENV1 NS5 (IC50 2 μM)) [8] DENV2 NS5 (IC50 1 μM) [7,8,11] Hendra V (IC50 15 μM) [13] SV40 T-ag [34]	IMPβ1 (IC50 7 μM) [11]
Cellular Context		
1) Inhibits binding of IMPα in transfected cell context (BiFC/CoIP)	SV40 T-ag [11,20] DENV2 NS5 ([7,11] Adenovirus EIA [20]	
2) Inhibits nuclear accumulation in a transfected cell context of IMPα/β1- (but not β1-) recognised proteins (CLSM) [7]	HIV-IN [7] SV40 T-ag [7,16,34] DENV2 NS5 [7] VEEV Capsid [16] adenovirus E1A [20] PSV UL42 [18] hCMV ppUL44, pUL5 [7]	TRF1 [7,34] p53 [7] NF-kB (LPS-induced) [37,38]
3) Reduces nuclear localisation in infected cells	VEEV Capsid [9] adenovirus E1A [20]	

[a]Abbreviations: HIV-1, human immunodeficiency virus; DENV, dengue virus; VEEV, Venezuelan equine encephalitis virus; PSV, Pseudorabies virus; hCMV, human cytomegalovirus; T-ag, SV40 large T-antigen; DENV, dengue virus; TRF1, telomere repeat factor 1; LPS, lipopolysaccharide.
[b]Inhibits helicase activity (FRET based assay) of DENV2, YFV and WNV NS3 (IC50 0.2–0.5 μM) [12].

Ivermectin's potential as an antiviral to treat infection can of course only be demonstrated in clinical studies. Preclinical studies in a lethal Pseudorabies (PRV) mouse challenge model showed that dosing (0.2 mg/kg) 12 h post-infection protected 50% of mice, which could be increased to 60% through administration of ivermectin at the time of infection [18]. Clinical data for a phase III trial against DENV infection similarly indicate antiviral activity, with daily dosing (0.4 mg/kg) found to be safe, and virological efficacy demonstrated, although it was concluded that dosing regimen modification was required to achieve clinical benefit [41]. These studies underline ivermectin's real potential as an antiviral able to reduce viral load in a clinical context.

Table 2
Documented antiviral action of ivermectin.[a]

Virus	Inhibitory Concentration/Fold reduction (Assay)
Coronavirus	
SARS-CoV-2	EC50 = 2.2/2.8 μM/5000-fold reduction (qPCR/released/cell-associated virus) [17]
Lentivirus	
HIV-1 (VSV-G-pseudotyped NL4-3.Luc.R-E-HIV)	50 μM > 2-fold reduction (luciferase) [7]
Orthomyxovirus	
Influenza VLPs (avian influenza A/MxA escape mutants)	10 μM total inhibition (luciferase) [10]
Flavivirus:	
YFV (17D)	EC50 = 5/0.5 nM (CPE/qPCR) [12]/3 μM > 50,000-fold reduction (pfu) [15]
DENV1 (EDEN-1)	EC50 = 2.3/3 μM (CFI, 2 hosts) [8]/EC50 = 0.7 μM (qPCR) [12]
DENV2 (NGC)	EC50 = 0.4/0.6 μM (pfu/qPCR) [11]/50 μM total inhibition (pfu) [7]
DENV2 (EDEN-2)	EC50 = 2.1/1.7 μM (CFI, 2 hosts) [8]
DENV3 (EDEN-3)	EC50 = 1.7 μM (CFI) [8]
DENV4 (EDEN-4)	EC50 = 1.9 μM (CFI) [8]
WNV (NY99)	EC50 = 4 μM (qPCR) [12]
WNV (MRM61C)	EC50 = 1/0.5 μM (pfu/qPCR) [11]
ZIKV (Asian/Cook Islands/2014)	EC50 = 1.3/1.6 μM (pfu/qPCR) [11]
Alphavirus:	
Chikungunya virus (CHIKV-Rluc)	EC50 = 1.9/0.6 μM (luciferase, 2 hosts)/3 μM > 5000-fold reduction (pfu) [15]
Sindbis (HR)	3 μM > 1000-fold (pfu) [15]
Semlicki Forest Virus	3 μM > 200-fold (pfu) [15]
VEEV (TC83)	1 μM c. 20-fold (pfu) [9]
Henipavirus:	
Hendra (Hendra virus/Australia/Horse/1994)	est. EC50 = 2 μM (TCID/luciferase) [13]
DNA viruses	
Adenovirus (HAdV-C5)	EC50 = c. 2.5 μM; 10 μM 20-fold reduction (qPCR) [20]
Adenovirus (HAdV-B3)	10 μM c. 8-fold reduction (qPCR) [20]
BK polyomavirus (BKPyV)	Est. EC50 1.5 μM (pfu/CPE/qPCR) [19]
Pseudorabies	Est. EC50 c. 0.8 μM 1000-fold [18]

[a]*In vitro*
[b]Inhibits helicase activity (FRET based assay) of DENV2, YFV and WNV NS3 (IC50 0.2–0.5 μM) [12].
Abbreviations: HIV-1, human immunodeficiency virus; VLP, virus like particle; PSV, Pseudorabies virus; YFV (yellow fever virus); DENV, dengue virus; ZIKV, zika virus; WNV, West Nile virus; TCID; pfu, plaque forming unit (infectious virus assay); CPE, cytopathic effects; qPCR, quantitative polymerase chain reaction; CFI, cell fluorescence-based immunofluorescence assay.

FOI 26-3132 - Document 5

Table 3
Summary of Current Clinical Trials using Ivermectin for SARS-CoV-2.

	Title, URL	Status[a]	N[b]	Interventions[c]	Start	Locations
1	Ivermectin Effect on SARS-CoV-2 Replication in Patients With COVID-19; https://ClinicalTrials.gov/show/NCT04381884	R	45	**Ivermectin** 0.6 mg/kg QD plus SC vs. SC	18.5.20	CEMIC, Buenos Aires, Ciudad De Buenos Aires, Argentina
2	Ivermectin and Nitazoxanide Combination Therapy for COVID-19; https://ClinicalTrials.gov/show/NCT04360356	NY	100	**Ivermectin** 0.2 mg/kg once plus NZX 500 mg BID for 6 days vs. SC	20.5.20	Tanta University, Egypt
3	Ivermectin vs. Placebo for the Treatment of Patients With Mild to Moderate COVID-19; https://ClinicalTrials.gov/show/NCT04429711	R	100	**Ivermectin** 12–15 mg/day for 3 days vs. Placebo	12.5.20	Sheba Medical Center, Ramat-Gan, Israel
4	Hydroxychloroquine and Ivermectin for the Treatment of COVID-19 Infection; https://ClinicalTrials.gov/show/NCT04391127	A	200	**Ivermectin** 12 mg (<80 kg) or 18 mg (>80 kg) once vs. HCQ 400 mg BID for 1 day then 200 mg BID for 4 days vs. Placebo	4.5.20	Jose Manuel Arreola Guerra, Aguascalientes, Mexico
5	Efficacy of Ivermectin in Adult Patients With Early Stages of COVID-19; https://ClinicalTrials.gov/show/NCT04405843	R	400	**Ivermectin** 0.3 mg/kg daily for 5 days vs. Placebo	20.6.20	Colombia
6	Ivermectin In Treatment of COVID 19 Patients; https://ClinicalTrials.gov/show/NCT04425707	R	100	**Ivermectin** (dose unlisted) vs. SC vs. **Ivermectin** (dose unlisted) plus SC	9.6.20	Isolation and referral hospitals for COVID 19 patients, Cairo, Egypt
7	Efficacy and Safety of Ivermectin and Doxycycline in Combination or IVE Alone in Patients With COVID-19 Infection; https://ClinicalTrials.gov/show/NCT04407130	E	72	**Ivermectin** 0.2 mg/kg once plus 200 mg DOC day 1 followed by 100 mg DOC BID for 4 days vs. **Ivermectin** 0.2 mg/kg QD for 5 days vs. Placebo	16.6.20	ICDDR, Dhaka, Bangladesh
8	Efficacy of Ivermectin as Add on Therapy in COVID19 Patients; https://ClinicalTrials.gov/show/NCT04343092	C	100	**Ivermectin** 0.2 mg/kg once weekly plus HCQ 400 mg QD plus ATM 500 mg QD vs. HCQ 400 mg QD plus ATM 500 mg QD	18.4.20	General Directorate of Medical City, Bagdad, Baghdad, Iraq
9	COVidIVERmectin: Ivermectin for Treatment of Covid-19 (COVER); https://ClinicalTrials.gov/show/NCT04438850	R	102	**Ivermectin** 0.6 mg/kg QD for 5 days vs. **Ivermectin** 1.2 mg/kg QD for 5 days vs. Placebo	20.6.20	Negrar, Verona, Italy; Bologna, Italy; Milan, Italy; Rovereto, Italy; Turin, Italy; Barcelona, Spain; Madrid, Spain
10	Efficacy, Safety and Tolerability of Ivermectin in Subjects Infected With SARS-CoV-2 With or Without Symptoms (SILVERBULLET); https://ClinicalTrials.gov/show/NCT04407507	NY	66	**Ivermectin** 12 mg/day for 3 days plus paracetamol 500 mg QID for 14 days vs. Placebo plus paracetamol 500 mg QID for 14 days	20.6.20	Investigacion Biomedica para el Desarrollo de Farmacos S.A. de C.V., Mexico
11	Sars-CoV-2/COVID-19 Ivermectin Navarra-ISGlobal Trial (SAINT); https://ClinicalTrials.gov/show/NCT04390022	A	24	**Ivermectin** 0.4 mg/kg once vs. Placebo	14.5.20	Clinica Universidad de Navarra, Pamplona, Navarra, Spain
12	A Comparative Study on Ivermectin and Hydroxychloroquine on the COVID19 Patients in Bangladesh; https://ClinicalTrials.gov/show/NCT04434144	C	116	**Ivermectin** 0.2 mg/kg once plus DOC 100 mg BID for 10 days vs. HCQ 400 mg day 1 then 200 mg BID for 9 days plus ATM 500 mg/day for 5 days	2.5.20	Chakoria Upazilla Health Complex, Cox's Bazar, Bangladesh
13	Ivermectin vs Combined Hydroxychloroquine and Antiretroviral Drugs (ART) Among Asymptomatic COVID-19 Infection (IDRA-COVID19); https://ClinicalTrials.gov/show/NCT04435587	NY	80	**Ivermectin** 0,6 mg/kg daily for 3 days vs. HCQ 400 mg BID Day 1 then 200 mg BID for 4 days plus Darunavir/ritonavir (400 mg/100 mg) BID for 5 days	20.7.20	Siriraj Hospital, Bangkok Noi, Bangkok, Thailand
14	IVERMECTIN Aspirin Dexametasone and Enoxaparin as Treatment of Covid 19; https://ClinicalTrials.gov/show/NCT04425863	A	100	**Ivermectin** 5 mg/ml oral to be repeated 1 week later (dose unlisted)	1.5.20	Hospital Eurnekian, Buenos Aires, Argentina
15	A Preventive Treatment for Migrant Workers at High-risk of Covid-19; https://ClinicalTrials.gov/show/NCT04446104	C	5000	**Ivermectin** 12 mg once vs. HCQ 400 mg day 1 then 200 mg/day for 42 days vs. Zinc 80 mg/day plus vitamin C 500 mg/day for 42 days vs. Povidone-iodine throat spray TID for 42 days vs. Vitamin C 500 mg/day for 42 days	13.5.20	Tuas South Dormitory, Singapore, Singapore
16	New Antiviral Drugs for Treatment of COVID-19; https://ClinicalTrials.gov/show/NCT04392427	NY	100	**Ivermectin** (dose unlisted) plus NZX (dose unlisted) plus ribavirin 200 mg or 400 mg vs. Control (untreated)	20.5.20	Mansoura University, Mansoura, Select A State Or Province, Egypt
17	Early Treatment With Ivermectin and LosarTAN for Cancer Patients With COVID-19 Infection (TITAN); https://ClinicalTrials.gov/show/NCT04447235	R	176	**Ivermectin** 12 mg once plus losartan 50 mg/day for 15 days vs. Placebo	20.7.20	Instituto do Cancer do Estado de São Paulo, Brazil
18	Ivermectin in Treatment of COVID-19; https://ClinicalTrials.gov/show/NCT04445311	R	100	**Ivermectin** daily (dose unlisted) for 3 days plus SC vs. SC	31.5.20	Waheed Shouman, Zagazig, Sharkia, Egypt
19	Efficacy of Ivermectin in COVID-19; https://ClinicalTrials.gov/show/NCT04392713	R	100	**Ivermectin** 12 mg once plus SC vs. SC	15.4.20	Combined Military Hospital Lahore, Lahore, Punjab, Pakistan
20	Ivermectin and Doxycycine in COVID-19 Treatment; https://ClinicalTrials.gov/show/NCT04403555	R	40	**Ivermectin** (dose unlisted) plus DOC (dose unlisted) vs. CQ (dose unlisted)	1.6.20	Sherief Abd-Elsalam, Tanta, Egypt
21	The Efficacy of Ivermectin and Nitazoxanide in COVID-19 Treatment; https://ClinicalTrials.gov/show/NCT04351347	R	300	**Ivermectin** (dose unlisted) vs. **Ivermectin** (dose unlisted) plus NZX (dose unlisted) vs. **Ivermectin** (dose unlisted) plus CQ (dose unlisted)	16.6.20	Tanta University, Tanta, Egypt
22	Prophylactic Ivermectin in COVID-19 Contacts; https://ClinicalTrials.gov/show/NCT04422561	C[d]	304	**Ivermectin** (dose unlisted) 2 doses 72 h apart vs. Control (untreated)	31.5.20	Zagazig University, Zagazig, Sharkia, Egypt
23	Max Ivermectin- COVID 19 Study Versus Standard of Care Treatment for COVID 19 Cases. A Pilot Study; https://ClinicalTrials.gov/show/NCT04373824	R	50	**Ivermectin** 0.2–0.4 mg/kg daily for 2 days plus SC vs. SC	25.4.20	Max Super Speciality Hospital, Saket (A unit of Devki Devi Foundation), New Delhi, Delhi, India

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FOI 26-3132 - Document 5

Table 3 (*continued*)

	Title, URL	Status[a]	N[b]	Interventions[c]	Start	Locations
24	A Study to Compare the Efficacy and Safety of Different Doses of Ivermectin for COVID-19 (IFORS); https://ClinicalTrials.gov/show/NCT04431466	R	64	**Ivermectin** 0.1 mg/kg once vs. **Ivermectin** 0.1 mg/kg day 1 and repeated after 72 h vs. **Ivermectin** 0.2 m/kg once vs. **Ivermectin** 0.2 mg/kg day 1 and repeated after 72 h vs. SC	1.7.20	Hospital Univeristário da Universidade Federal de São Carlos (HU-UFSCar), São Carlos, São Paulo, Brazil
25	Novel Agents for Treatment of High-risk COVID-19 Positive Patients; https://ClinicalTrials.gov/show/NCT04374019	R	240	**Ivermectin** 12 mg (<75 kg) or 15 mg (>75 kg) daily for 2 days vs. HCQ 600 mg/day for 14 days plus ATM 500 mg day 1 then 250 mg/day for 4 days vs. Camostat Mesilate 200 mg TID for 14 days vs. Artemesia annua 50 mg TID for 14 days	1.5.20	University of Kentucky Markey Cancer Center, Lexington, Kentucky, United States
26	Ivermectin-Azithromycin-Cholecalciferol (IvAzCol) Combination Therapy for COVID-19; https://ClinicalTrials.gov/show/NCT04399746	C[e]	30	**Ivermectin** 6 mg/day on days 0, 1, 7 and 8 plus ATM 500 mg/day 4 days plus Cholecalciferol 400 IU BID for 30 days vs. Control (untreated)	15.3.20	Outpatient treatment, Mexico City, Mexico
27	USEFULNESS of Topic Ivermectin and Carrageenan to Prevent Contagion of Covid 19 (IVERCAR); https://ClinicalTrials.gov/show/NCT04425850	A[f]	1195	**Ivermectin** (topical for oral mucosae) plus iota carrageenan (topical for oral mucosae) 5 times per day plus PPE vs. PPE only	1.6.20	Hospital Eurnekian, Buenos Aires, Argentina
28	Novel Regimens in COVID-19 Treatment; https://ClinicalTrials.gov/show/NCT04382846	NY	80	**Ivermectin** plus CQ (dose unlisted) vs. **Ivermectin** plus NZX (dose unlisted) vs. **Ivermectin** plus NZX plus ATM (dose unlisted) vs. NZX and ATM (dose unlisted)	8.5.20	Tanta University, Egypt
29	Anti-Androgen Treatment for COVID-19; https://ClinicalTrials.gov/show/NCT04446429	R	254	**Ivermectin** 0.2 mg/kg QD plus ATM 500 mg QD vs. **Ivermectin** 0.2 mg/kg QD plus ATM 500 mg QD plus Dutasteride 0.5 mg QD	26.6.20	Corpometria Institute, Brasilia, Brazil
30	A Real-life Experience on Treatment of Patients With COVID 19; https://ClinicalTrials.gov/show/NCT04345419	R	120	**Ivermectin** (dose unlisted) vs. CQ (dose unlisted) vs. Favipiravir (dose unlisted) vs. NZX (dose unlisted) vs. Niclosamide (dose unlisted) vs. other drugs (oseltamivir or combination of above, dose unlisted)	16.6.20	Tanta University Hospital, Tanta, Egypt
31	Worldwide Trends on COVID-19 Research After the Declaration of COVID-19 Pandemic (observational); https://ClinicalTrials.gov/show/NCT04460547	NY	200	Completed interventional vs. completed observational studies on **Ivermectin**, Convalescent Plasma, HCQ, DAS181, or Interferon β1A	25.7.20	Qassim University, Saudi Arabia
32	Trial of Combination Therapy to Treat COVID-19 Infection; https://ClinicalTrials.gov/show/NCT04482686	NY	300	**Ivermectin** (dose unlisted) day 1 and 4 plus DOC (dose unlisted) for 10 days plus Zinc for 10 days plus Vitamin D3 for 10 days plus Vitamin C for 10 days vs. Placebo	22.7.20	ProgenaBiome, California, USA
33	Randomised clinical trial of ivermectin for treatment and prophylaxis of COVID-19; https://www.clinicaltrialsregister.eu/ctr-search/trial/2020-001994-66/ES	O	266	**Ivermectin** (dose unlisted) vs. Placebo	8.5.20	Fundació Assistencial Mútua Terrassa, Spain
34	Multicenter, randomized, double-blind, placebo-controlled study investigating efficacy, safety and tolerability of ivermectin HUVE-19 in patients with proven SARS-CoV-2 infection (COVID-19) and manifested clinical symptoms; https://www.clinicaltrialsregister.eu/ctr-search/trial/2020-002091-12/BG	O	120	**Ivermectin** 0.4 mg/kg plus SC vs. Placebo plus SC	5.5.20	Bulgaria (9 sites)
35	Efficacy of hydroxychloroquine, ciclesonide and ivermectin in treatment of moderate covid-19 illness: an open-label randomised controlled study (EHYCIVER-COVID); http://ctri.nic.in/Clinicaltrials CTRI/2020/04/024948	NY	120	**Ivermectin** 12 mg/day for 7 days vs. Ciclesonide 0.2 mg/kg BID for 7 days vs. HCQ 400 mg BID Day 1 then 200 mg BID for 6 days vs. SC	15.5.20	New Delhi, India
36	A Phase IIB open label randomized controlled trial to evaluate the efficacy and safety of Ivermectin in reducing viral loads in patients with hematological disorders who are admitted with COVID 19 infection; http://ctri.nic.in/Clinicaltrials CTRI/2020/04/025068	NY	50	**Ivermectin** 3 mg (15-24 kg) or 6 mg (25-35 kg) or 9 mg (36-50 kg) or 12 mg (51-65 kg) or 15 mg (66-79 kg) or 0.2 mg/kg (80 kg) once vs. SC	27.5.20	Christian Medical College Vellore, Tamil Nadu, India
37	Interventional study to assess the efficacy of Ivermectin with standard of care treatment versus standard of care in patients of COVID-19 at R D Gardi Medical College, Ujjain, India; http://ctri.nic.in/Clinicaltrials CTRI/2020/04/025224	NY	50	**Ivermectin** 12 mg/day for 2 days plus SC vs. SC	24.5.20	R D Gardi Medical College, Ujjain, Madhya Pradesh, India
38	Study to assess the efficacy of Ivermectin as prophylaxis of COVID 19 among health care workers and COVID 19 contacts in Ujjain, India; http://ctri.nic.in/Clinicaltrials CTRI/2020/04/025333	NY	2000	**Ivermectin** 12 mg/day (adult) or 6 mg/day (children) for 2 days vs. Control	27.5.20	R D Gardi Medical College, Ujjain, Madhya Pradesh, India
39	Randomised Controlled Trial of Ivermectin in hospitalised patients with COVID19 (RIVET-COV); http://ctri.nic.in/Clinicaltrials CTRI/2020/04/026001	NY	60	**Ivermectin** single dosing of 0.2 mg/kg vs. **Ivermectin** 0.4 mg/kg vs. **Ivermectin** 0.8 mg/kg vs. **Ivermectin** 1.6 mg/kg vs. **Ivermectin** 2 mg/kg vs. SC	25.6.20	New Delhi, India
40	A Prospective, randomized, single centred, open labelled, two arm, placebo-controlled trial to evaluate efficacy and safety of Ivermectin drug in patients infected with SARS-CoV-2 virus; http://ctri.nic.in/Clinicaltrials CTRI/2020/04/025960	NY	100	**Ivermectin** 12 mg/day for 3 days vs. SC	18.6.20	Symbiosis University Hospital and Research Centre, Maharashtra, India
41	A Clinical Trial to Study the Efficacy of "Ivermectin" in the prevention of Covid-19. A Single Arm Study; http://ctri.nic.in/Clinicaltrials CTRI/2020/04/026232	NY	50	**Ivermectin** 0.2 mg/kg once	10.7.20	DVFM, Andhra Pradesh, India

FOI 26-3132 - Document 5

Table 3 (*continued*)

	Title, URL	Status[a]	N[b]	Interventions[c]	Start	Locations
42	Ivermectin Nasal Spray for COVID19 Patients; https://ClinicalTrials.gov/show/NCT04510233	NY	60	**Ivermectin** nasal spray (1 ml) in each nostril BID vs. **Ivermectin** oral (6 mg) TID vs. SC	10.8.20	Tanta University, Tanta, Egypt
43	Outpatient use of ivermectin in COVID-19; https://ClinicalTrials.gov/show/NCT04530474	NY	200	**Ivermectin** 0.15–0.2 mg/kg (max 12 mg) once vs. Placebo	26.8.20	Temple University Hospital, Philadelphia, USA
44	Ivermectin to prevent hospitilizations in COVID-19; https://ClinicalTrials.gov/show/NCT04529525	R	500	**Ivermectin** 12 mg (48-80 kg) or 18 mg (80-110 kg) or 24 mg (>100 kg) at inclusion and again at 24h vs. Placebo	21.8.20	Ministry of Public Health, Province of Corrientes, Argentina
45	Clinical trial of ivermectin plus doxycycline for the treatment of confirmed Covid-19 infection; https://ClinicalTrials.gov/show/NCT04523831	C[g]	400	**Ivermectin** 6 mg and DOC 100 mg BID for 5 days vs. Placebo	19.8.20	Dhaka Medical College, Dhaka Bangladesh
46	Pilot study to evaluate the potential of ivermectin to reduce COVID-19 transmission; https://www.clinicaltrialsregister.eu/ctr-search/trial/2020-001474-29/ES	O	24	**Ivermectin** (dose unlisted) vs. Placebo	8.5.20	Clinica Universidad de Navarra, Pamplona, Spain
47	Dose-Finding study of Ivermectin treatment on patients infected with Covid-19:A clinical trial; https://en.irct.ir/trial/47012	A	125	**Ivermectin** 0.2 mg/kg single dose plus SC vs. **Ivermectin** 0.2 mg/kg day 1, 2, 5 plus SC vs Placebo plus SC vs. **Ivermectin** 0.4 mg/kg day 1 and 0.2 mg/kg day 2, 5 vs. SC	4.5.20	Qazvin University of Medical Sciences, Qazvin, Iran
48	In vivo use of ivermectin (IVR) for treatment for corona virus infected patients: a randomized controlled trial; http://www.chictr.org.cn/showprojen.aspx?proj=54707	NY	60	**Ivermectin** single dose 0.2 mg/kg vs. Placebo	10.6.20	Rayak Hospital, Riyaq, Lebanon
49	A randomized clinical trial study, comparison of the therapeutic effects of Ivermectin, Kaletra and Chloroquine with Kaletra and Chloroquine in the treatment of patients with coronavirus 2019 (COVID-19); http://en.irct.ir/trial/48444	A	60	**Ivermectin** 0.15–0.2 mg/kg single dose day 1 plus HCQ 200 mg day 1 plus Lopinavir/Ritonavir 400/100 mg days 2–6 vs. HCQ 200 mg day 1 plus Lopinavir/Ritonavir 400/100 mg days 2–6	30.5.20	Ahvaz Razi Hospital, Ahvaz, Iran
50	A double-blind clinical trial to repurpose and assess the efficacy and safety of ivermectin in COVID-19; http://isrctn.com/ISRCTN40302986	R	45	**Ivermectin** 6 mg every 3.5 days for 2 weeks vs. **Ivermectin** 12 mg every 3.5 days for 2 weeks vs. Placebo	23.4.20	Lagos University Teaching Hospital, Lagos, Nigeria
51	Effectiveness of Ivermectin in the Treatment of Coronavirus Infection in Patients admitted to Educational Hospitals of Mazandaran in 2020; https://en.irct.ir/trial/49174	R	60	**Ivermectin** 0.2 mg/kg single dose plus SC vs. SC	21.5.20	Bouali Hospital, Sari, Iran
52	Sub-cutaneous Ivermectin in Combination With and Without Oral Zinc and Nigella Sativa: a Placebo Randomized Control Trial on Mild to Moderate COVID-19 Patients; https://clinicaltrials.gov/ct2/show/study/NCT04472585	R	40	**Ivermectin** 0.2 mg/kg subcutaneous injection every 2 days plus SC vs. **Ivermectin** 0.2 mg/kg subcutaneous injection every 2 days plus 80 mg/kg Nigella Sativa oral QD plus SC vs. **Ivermectin** 0.2 mg/kg subcutaneous injection every 2 days plus 20 mg Zinc Sulfate oral TID plus SC vs. Placebo plus SC	14.7.20	Shaikh Zayed Hospital, Lahore, Pakistan
53	Pragmatic study "CORIVER": Ivermectin as antiviral treatment for patients infected by SARS-COV2 (COVID-19); https://www.clinicaltrialsregister.eu/ctr-search/trial/2020-001971-33/ES	O	45	**Ivermectin** 0.2–0.4 mg/kg (regime unlisted) vs. HCQ 400 mg vs ATM 500 mg vs. Placebo	22.7.20	Hospital Universitario Virgen de las Nieves, Granada, Spain
54	Effectiveness and Safety of Ivermectin for the Prevention of Covid-19 Infection in Colombian Health Personnel at All Levels of Care, During the 2020 Pandemic: A Randomized Clinical Controlled Trial; https://clinicaltrials.gov/ct2/show/record/NCT04527211	NY	550	**Ivermectin** 0.2 mg/kg weekly for 7 weeks vs. Placebo	7.9.20	Pontificia Universidad Javeriana, Valle Del Cauca, Colombia
55	Ivermectin Inhalation Forms in the Management of COVID-19 Egyptian Patients; https://clinicaltrials.gov/ct2/show/NCT04510233	NY	60	**Ivernectin** nasal spray BID (dose unlisted) vs. **Ivermectin** oral 6 mg TID vs. SC	10.8.20	Tanta University, Tanta, Egypt
56	Safety and Efficacy of Ivermectin and Doxycycline in Treatment of Covid-19. https://clinicaltrials.gov/ct2/show/NCT04551755	NY	188	**Ivermectin** 12 mg first dose then 12 mg after 12 h plus DOC 100 mg BID for 10 days vs. Placebo	16.9.20	Bangladesh Medical College Hospital, Dhaka, Bangladesh
57	Comparative Study of Hydroxychloroquine and Ivermectin in Covid-19 Prophylaxis; https://clinicaltrials.gov/ct2/show/NCT04384458	R	400	**Ivermectin** (dose based on weight, unlisted) QD for 2 days repeated every 14 days for 45 days plus 20 mg BID active Zinc versus HCQ 400 mg BID on day 1, and QD on days 2–5 followed by QD every 5 days for 50 days plus 20 mg BID active Zinc	12.5.20	Drug Research and Development Centre, Federal University of Ceará, Ceará, Brazil
58	Assessment of response of ivermectin on virological clearance in COVID 19 patients; http://ctri.nic.in/Clinicaltrials CTRI/2020/08/027394	NY	56	**Ivermectin** 0.2 mg/kg single dose vs. SC	26.08.20	Maulana Azad Medical College, New Delhi, India
59	Evaluation of the effect of ivermectin on patients with COVID-19; http://en.irct.ir/trial/50305	C	130	**Ivermectin** 0.2 mg on day 1 followed by 3 mg BID days 2–4 plus HCQ sulfate and ATM (both according to protocol of Ministry of Health) vs. HCQ sulfate and ATM alone	23.08.20	Tehran University of Medical Sciences, Tehran, Iran
60	Prophylactic Ivermectin in COVID 19 Contacts http://ctri.nic.in/Clinicaltrials; CTRI/2020/08/027282	NY	180	**Ivermectin** 12 mg once vs. **Ivermectin** 36 mg once vs. Placebo	20.08.20	Government Institute of Medical Sciences Greater Noida, Uttar Pradesh, India

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FOI 26-3132 - Document 5

Table 3 (*continued*)

	Title, URL	Status[a]	N[b]	Interventions[c]	Start	Locations
61	Ivermectin as a possible treatment for COVID-19; http://ctri.nic.in/Clinicaltrials CTRI/2020/08/027225	NY	90	**Ivermectin** 12 mg QD on days 1–2 vs. Placebo	18.08.20	AAIMS, Patna, India
62	Evaluating the effect of Ivermectin on covid 19 patients; http://en.irct.ir/trial/49935	R	60	**Ivermectin** 14 mg every 12 h for 36 h then again on day 7 vs. Placebo	06.08.20	Ahvaz University of Medical Sciences, Ahvaz, Iran
63	Evaluate the Efficacy of Siddha Treatment in Patients with Novel Coronavirus Infectious Disease; http://ctri.nic.in/Clinicaltrials CTRI/2020/08/026999	R	100	**Ivermectin** 12 mg once plus DOC 100 mg BID for 5 days plus Vitamin C 500 mg QD plus Zinc QD plus SC vs. Siddha traditional medicine protocol for 7 days	05.08.20	Indian Medicine and Homeopathy Department, Tamil Nadu, India
64	Ivermectin effect in the treatment of patients with covid-19; http://en.irct.ir/trial/49180	R	40	**Ivermectin** 0.2 mg/kg QD for 2 days vs. antiviral drugs (eg. HCQ) as per SC	20.07.20	Mashhad University of Medical Sciences, Mashhad, Iran
65	Randomized phase IIA clinical trial to compare the efficacy of ivermectin versus placebo to obtain negative PCR results in patients with early phase Covid-19; PER-034-20	R	68	**Ivermectin** 0.3 mg/kg QD for 3 days vs. Placebo	17.07.20	Universidad Peruana Cayetano Heredia, Lima, Peru
66	Evaluation of ivermectin effects on Covid-19; http://en.irct.ir/trial/49280	R	50	**Ivermectin** 0.15 mg/kg/day plus SC vs. SC	22.08.20	Kermanshah University of Medial Sciences, Kermanshah, Iran
67	A placebo-controlled, randomized, double-blind study in COvid-19 patients with iveRmectin; An inVEstigator iniTiaTEd trial (CORVETTE-01); jRCT2031200120	R	240	**Ivermectin** 0.2 mg/kg once vs. Placebo	16.09.20	Kitasato University Hospital, Kanagawa, Japan
68	A single-centre, open-label, randomized controlled study of ivermectin treated mild to moderate COVID-19 cases; Debidwar Upazila Health Complex	C[h]	62	**Ivermectin** 0.2 mg/kg once plus SC vs. SC	01.05.20	Debidwar Upazila Health Complex, Comilla, Bangladesh

[a] R, Recruiting, NY, Not yet recruiting, A, Active not recruiting, C, Completed, E, Enrolling by invitation, O, ongoing.
[b] Number of patients.
[c] SC, standard care, QD, once per day, BID, twice daily, QID, 4 times daily, TID, 3 times daily, PPE, personal protective equipment, vs. versus, HCQ, hydroxychloroquine; DOC, doxycycline; CQ, chloroquine, ATM, Azithromycin, NZX, Nanozoxide
[d] Raw data for asymptomatic family close contacts of confirmed COVID patients show that 2 doses of ivermectin 72 h apart resulted in only 7.4% of 203 subjects reporting symptoms of SARS-CoV-2 infection, in contrast to 101 control untreated subjects, of whom 58.4% reported symptoms; evidence of prophylaxis by ivermectin.
[e] Recovery rate of the 28 patients that received ivermectin/AZM/cholecalciferol was 100%, with mean symptomatic recovery 3.6 days (negative PCR confirmed day 10). Imaging on day 10 showed improvement in all patients with pneumonia. Authors conclude the combination therapy might mitigate disease progression without significant adverse effects but further studies required (preferably controlled) [46].
[f] Preliminary results for 1195 subjects consistent with prophylaxis effected by ivermectin/carrageenan topical combination [47].
[g] Raw data shows a significant reduction in the number of 183 patients with late clinical recovery (requiring >12 days to show clinical improvement) in the ivermectin/DOC group compared to placebo (23 versus 37.2%), as well as a significant reduction (8.7 versus 17.8%) in patients showing clinical deterioration (from mild/moderate to moderate or severe), and a significant reduction (7.7 versus 20%) in persistent Covid-19 positive patients at 14 days compared to 180 control patients; evidence of efficacy for ivermectin/DOC.
[h] No statistically significant clinical benefit in 32 treated subjects compared to 30 subjects given placebo, but authors concluded study requires confirmation with larger numbers of subjects [48].

6. Ivermectin as a therapeutic for SARS-CoV-2 infection ?

The current SARS-CoV-2 pandemic has eclipsed the porcine flu epidemic in terms of numbers of infections (currently >70 million) and deaths (>1.6 million, with >310,000 of these in the US alone) worldwide. The search for antivirals through repurposing existing drugs has proved challenging (eg. see Ref. [42,43]), one important aspect of repurposing being the perceived need to achieve therapeutic levels in the lung. Published pharmacokinetic modelling based on both the levels of ivermectin achievable in human serum from standard parasitic treatment dosing and robust large animal experiments where lung levels of ivermectin can be measured, indicates that concentrations of ivermectin 10 times higher than the c. 2.5 μM EC_{50} indicated by *in vitro* experiments (Table 2) are likely achievable in the lung in the case of SARS-CoV-2 [39]; modelling based on different assumptions predicts lower values, but highlights the long-term stability of ivermectin in the lung (for over 30 days) based on data from animals [40]. It should also be noted that liquid formulations for intravenous administration of long-acting ivermectin have been described, with aerosol administration also in development, to enable ivermectin administration to achieve even higher concentrations to tackle SARS-CoV-2, whilst the use of ivermectin in combination with other agents may enhance efficacy at lower doses.

Ivermectin as a treatment for SARS-CoV-2 in humans has already been approved in a number of states and countries, including the Republic of Peru [44] and Northeastern Beni region of Bolivia [45]. Importantly, close to 70 trials worldwide are currently testing the clinical benefit of ivermectin to treat or prevent SARS-CoV-2 (see Table 3); these include variations on dosing regimens, combination therapies (preliminary results for NCT04523831 in Table 3, #45) [46,47], and prophylactic protocols. With respect to the latter, preliminary results from recently completed study NCT04422561 (Table 3, #22, and footnote) examining asymptomatic family close contacts of confirmed COVID patients, reveal that two doses of ivermectin 72 h apart result in only 7.4% of 203 subjects reporting symptoms of SARS-CoV-2 infection, in stark contrast to control untreated subjects, of whom 58.4% reported symptoms, underlining ivermectin's potential as a prophylactic. Results from retrospective/observational trials [48–52] are also consistent with clinical benefit. Mymensingh Medical College Hospital (Bangladesh) reported that none of 115 subjects receiving a single 12 mg dose of ivermectin developed pneumonia/cardiovascular complications, compared to 9.8% (pneumonia) and 1.5% (ischemic stroke) in 133 control subjects [49]. Further, significantly fewer ivermectin group patients developed respiratory distress (2.6 versus 15.8%), or required oxygen (9.6 versus 45.9%), antibiotics (15.7 versus 60.2%) or intensive care management (0.09 versus 8.3%). Ivermectin-treated patients became SARS-CoV-2 negative significantly faster (median 4 compared to 15 days), had significantly shorter hospital stays (median 9 versus 15 days), and significantly lower mortality (0.9 vs 6.8%). Although combinations of therapies were used, the results for a 196 patient propensity-matched cohort study (ICON - Ivermectin in COvid Nineteen – study) at Broward Health Medical Centre (Florida, USA) [50] indicate significantly reduced mortality (13.3%) in subjects receiving 0.2 mg/kg ivermectin (optional second dose 8 days later), compared to 24.5% mortality in those not receiving ivermectin, with more

FOI 26-3132 - Document 5

significant differences for patients with severe pulmonary involvement (mortality rates of 38.8 versus 80.7%). Although these early results are consistent with efficacy, it is clear that only the results from large rigorous randomized clinical trials (Table 3) will definitively establish ivermectin's utility to treat or prevent SARS-CoV-2 infection. It is to be hoped that the results from these trials will emerge in the next few months to document ivermectin's credentials or otherwise as a viable therapeutic for COVID-19 infection, and potentially infection by many other viruses.

Author contributions

Conceptualization, D.A.J.; writing—original draft preparation, D.A.J., K.M.W.; review and editing, D.A.J., K.M.W. Both authors have read and agreed to the published version of the manuscript.

Funding

We acknowledge financial support from the National Health and Medical Research Council Australia (Senior Principal Research Fellowship APP1002486/APP1103050) to D.A.J. and from the National Breast Cancer Foundation (ECF-17-007) to K.M.W.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Andrew Owen (University of Liverpool, United Kingdom), Joe Mymryk (University of Western Ontario, Ontario) and Anudeep Talagavadi Channaiah (Topiwala National Medical College & B. Y. L. Nair Charitable Hospital, India), are thanked for sharing recent publications.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Clinical Medicine: Therapeutics

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REVIEW

Safety and Efficacy Review of Doxycycline

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Abstract: Doxycycline is a member of the tetracycline class of antibiotics and has been used clinically for more than 40 years. It is a well-tolerated drug that is bacteriostatic and acts via the inhibition of bacterial ribosomes. It is generally given at a dose of 100-mg daily or twice daily. It is well absorbed and has generally good tissue penetration. The serum half-life is 18–22 hours and dosage does not need to be adjusted in the presence of renal or hepatic impairment. Major side effects are gastro-intestinal and dermatological and it is generally contra-indicated in pregnancy or childhood because of concerns about discolouration of developing teeth and potential effects on growing bones. Drug interactions are not common although can occur with the concomitant use of methotrexate and the oral contraceptive pill, and its absorption can be reduced by the co-administration with some antacids and iron preparations. It has activity against many organisms, including Gram-positives, Gram-negatives and atypical bacteria. In addition, it appears to have some potentially clinically useful anti-inflammatory properties.

Keywords: doxycycline, tetracyclines, antibiotics, infections

Clinical Medicine: Therapeutics 2009:1 471–482

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FOI 26-3132 - Document 5

Introduction

Tetracycline antibiotics were one of the first classes developed following the introduction of penicillin G and the sulphonamides. Doxycycline (alpha-6-deoxytetracycline; see Fig. 1) was developed by Pfizer and is a semi-synthetic derivative of oxytetracycline that first became available in 1967. It has the advantage over other members of the tetracycline family of improved oral absorption and a prolonged serum half-life. Doxycycline has activity against a very broad range of Gram-positive, Gram-negative and 'atypical' bacteria as well as some protozoa such as malaria. It is also a useful antibiotic for prophylaxis against and treatment of several important potential biological warfare agents. As such it is widely used in many parts of the world, in particular for sexually transmitted infections, respiratory tract infections, malaria prophylaxis and for the therapy of some arthropod-borne rickettsial infections.

Mechanism of Action

Doxycycline inhibits bacterial protein synthesis by reversibly binding to the 30S ribosomal subunit and preventing the association of aminoacyl-tRNA with the bacterial ribosome. Further inhibition of protein synthesis occurs in mitochondria through binding to the 70S ribosomes. It is therefore a bacteriostatic drug. Doxycycline enters the cell via hydrophilic pores in the outer cell membrane and a pH-dependent active transport system in the inner cytoplasmic membrane.[1] It also inhibits apicoplast ribosomal subunits in *Plasmodium falciparum*, leading to impaired fatty acid synthesis and impaired heme biosynthesis late in the malarial cell cycle.[2,3]

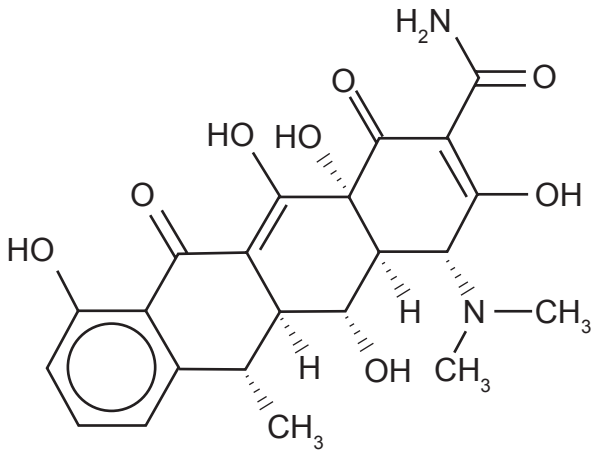


Figure 1. Chemical structure of doxycycline.

In addition, it has multiple other actions including the prevention of angiogenesis and apoptosis, enhancement of gingival fibroblast attachment, and wound healing.[4–6] It is known to inhibit certain matrix metalloproteases (MMPs), which are proteolytic enzymes produced by inflammatory cells. This has led to potential use in various anti-inflammatory and anti-neoplastic roles.[7,8] Subantimicrobial doses of doxycycline in periodontitis inhibit collagen degradation and the action of destructive MMPs in the gingivae.[9,10]

Pharmacokinetics and Pharmacodynamics

Doxycycline is almost completely absorbed following oral administration in the stomach and proximal small bowel. Food or dairy products do not significantly alter absorption, unlike for tetracycline and minocycline, with serum levels only falling by 20%.[11,12] It forms complexes with metal ions in food which are unstable in the acidic conditions in the stomach, and doxycycline enters the small bowel as free drug. However metal complexes formed in the duodenum are stable so a small amount of doxycycline is not absorbed. The presence of multivalent cations will impair doxycycline absorption.

There is a prolonged serum half-life of 18–22 hours, and this is not altered by impaired renal function. Peak serum levels occur 2–3 hours after oral administration and within 30 minutes after intravenous administration.[12,13] A single dose of oral doxycycline 200 mg achieves peak serum concentrations of approximately 3.0–5.0 µg/mL, and a single dose of intravenous doxycycline at the same dose achieves peak serum concentrations of 4–10 µg/mL.[12–14]

Penetration occurs in body fluids and tissues. Doxycycline is more lipid soluble than tetracycline, so high levels are detected in a range of tissues including lymphatic fluid, peritoneal fluid, colonic tissue, prostate tissue, and breast milk. Doxycycline penetrates more readily into bacterial cells compared with other tetracyclines.[15] Doxycycline also penetrates into cerebrospinal fluid with mean levels of 0.37–1.3 µg/mL or 14%–26% of serum concentration.[16–18] Concentrations are highest in excretory organs including the biliary system.[19] There is poor concentration in saliva and sputum.[20,21] Accumulation

occurs in teeth and bones via the formation of a stable calcium complex and leads to discolouration of teeth. Doxycycline also crosses the placenta, achieving umbilical cord plasma concentrations of 54% of maternal serum concentration.[22] The topical application of doxycycline to gingivae leads to detectable levels in gingival crevicular fluid but not serum.[23] Although oral doxycycline may reduce symptoms of ocular surface diseases (for example, meibomian gland dysfunction), it is not detectable in tear fluid samples.[24]

There is variable protein binding, reported up to 82%–90%.[25,26] The metabolism of doxycycline and other tetracyclines has not been investigated. More than one-third of doxycycline is excreted by the kidneys via glomerular filtration,[14,26,27] and a small percentage is excreted in the bile.[26,27] The remainder of doxycycline is excreted in faeces. In the presence of renal impairment, more doxycycline is excreted in faeces so that the same dose of doxycycline can be given without drug accumulation.[26–28] More than 90% of an oral dose of doxycycline is eliminated from the body within 72 hours of drug administration.

There are no data on the impact of sex, pregnancy, breast-feeding or hepatic impairment on pharmacokinetics and there are limited data available on doxycycline pharmacodynamics.[19] Concentration-dependent killing or area under the curve (AUC) is considered the significant parameter for doxycycline activity however there are no AUC/MIC targets. Doxycycline exerts a post-antibiotic effect (PAE) that is concentration dependent against a variety of organisms.[29] The significance of this is uncertain because adequate serum concentrations are maintained during the dosing interval if given on a daily or twice-daily regimen.[29]

Drug Interactions

Absorption is impaired by ferrous sulfate, bismuth and other antacids containing aluminium, calcium and magnesium salts.[13,30–34] Doxycycline should be given 2 hours prior to or 3 hours after iron supplementation.[13] It is not reduced by concomitant ranitidine.[35] Cytochrome P450 3A4 inducers such as rifampicin will reduce serum levels due to increased hepatic metabolism.[36] Barbiturates, anticonvulsants (phenytoin, carbamazepine), sodium bicarbonate, and acetazolamide will also lower plasma doxycycline levels.[37,38] Tetracyclines may lower plasma prothrombin activity, so patients on anticoagulant therapy may require reduction of their anticoagulant dosage.[39] The concurrent use of tetracycline and methoxyflurane has been reported to result in fatal renal toxicity.[40] The efficacy of oral contraceptives is impaired by concurrent doxycycline, so barrier contraception must be used during doxycycline therapy and for seven days after completion of therapy.[41–43] It may also lead to breakthrough inter-menstrual bleeding. Doxycycline should be used with caution in patients with chronic alcoholism as the half-life of doxycycline is reduced.[44] Doxycycline also may displace methotrexate from its binding site, leading to elevated methotrexate levels and toxicity.[34,45] Synergistic or additive effects with beta-lactams have been demonstrated experimentally with *Stenotrophomonas maltophilia* and *Chlamydophila trachomatis* with uncertain clinical significance.[46,47]

Dosage and Administration

Doxycycline is usually commenced with a starting dose of 200-mg daily, followed by a maintenance dose of 100-mg daily (or twice-daily for severe infections). It can be administered orally or intravenously. The oral dose should be taken with sufficient fluids and the patient should remain upright for 30 minutes following administration. The maximum recommended dose is 300-mg daily.[34] In children, when doxycycline benefits outweigh the risks, a weight-adjusted dose of 2.2 mg/kg daily or twice daily is used. A higher loading dose of doxycycline, for example 200-mg twice daily for 72 hours, is recommended for optimal dose-dependent killing in serious infections.[48] Variations exist with dosing and duration for specific indications, for example, malaria prophylaxis, syphilis, and scrub typhus. Lower doses, for example 20-mg twice daily, are used for acne vulgaris and rosacea. Topical preparations are also available for periodontitis.

Clinical Uses

Doxycycline has a broad-spectrum of activity against many Gram-positive, Gram-negative and 'atypical' bacteria, as seen in Table 1. As a result, it has many potential uses.

Respiratory infections

Doxycycline is frequently used in some parts of the world to treat respiratory tract infections such as community-acquired pneumonia (CAP) and acute

FOI 26-3132 - Document 5

Table 1. Doxycycline activity against selected pathogens.

Organism	Typical MIC_{90} µg per ml	Range
Gram-negative bacteria		
Escherichia coli	32	0.5–>64
Klebsiella pneumoniae	64	1–>64
Yersinia pestis	1	0.125–2
Haemophilus influenzae	1	0.5–8
Moraxella catarrhalis		0.1–4
Burkholderia pseudomallei	1.5	0.125–4
Stenotrophomonas maltophilia	2	0.5–4
Brucella melitensis	0.064	0.0156–0.094
Bartonella spp.	0.12	<0.016–0.25
Neisseria meningitides	1	0.12–2
Gram-positive bacteria		
Staphylococcus aureus	4	0.03–>16
Streptococcus pyogenes	8	0.1–>16
Streptococcus pneumoniae	8	0.04–16
Enterococcus faecalis	32	0.12–>64
Listeria monocytogenes	0.25	0.125–1
Bacillus anthracis	0.063	0.031–0.125
Nocardia spp.	16–32	0.125–32
Tropheryma whipplei		0.25–2
Other bacteria		
Legionella pneumophila	2	0.5–2
Mycoplasma pneumoniae	0.25	0.06–0.25
Chlamydophila pneumoniae	0.25	0.06–0.5
Chlamydophila trachomatis	0.25	0.125–0.25
Rickettsia spp.		0.06–0.125
Coxiella bunetii		2–4

exacerbations of chronic bronchitis (AECB). Its activity against 'atypical' bacteria such as *Mycoplasma pneumoniae*, *Chlamydophila* spp., *Legionella* spp. and *Coxiella burnetii* is reliable although resistance rates for 'typical' bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* are variable. Pneumococcal resistance rates for doxycycline range from 5%–25%.[49–51] Thus, in areas where resistance rates are either high or unknown, doxycycline should be combined with another agent that has more reliable anti-pneumococcal activity, such as a beta-lactam. In Australian patients with CAP treated with doxycycline combined with either benzylpenicillin, amoxycillin or ceftriaxone, very good outcomes were obtained,[52] and outcomes have been good even for confirmed cases of legionellosis.[53] Recent randomized studies comparing doxycycline with other agents are not available. In the CAP guidelines from the Infectious Diseases Society of America/American Thoracic Society, doxycycline is listed as one of the options for treating ambulatory patients with CAP who have no risk factors for drug-resistant pneumococcal infections.[54]

Doxycycline is generally listed as one of the first-line treatment options for AECB. Recent data for this is limited but in one study, it was superior to cefaclor and equivalent to amoxicillin, cephalexin and enoxacin.[55] Another small study compared it to roxithromycin and

found slightly lower rates of mortality and adverse events in the roxithromycin group.[56]

Genitourinary infections

For many years, seven-day courses of doxycycline were the treatment of choice for non-gonococcal urethritis (NGU) because of its reliable activity against *Chlamydophila* spp. With the availability of azithromycin, which can be given as a single oral dose to improve adherence, doxycycline use has decreased for this indication. Both drugs achieve similar high rates of successful outcomes of at least 97% for NGU due to *Chlamydophila trachomatis*.[57] Activity of doxycycline against *Ureaplasma urealyticum* is also good although occasional resistant strains are seen.[58] These same organisms can cause chronic prostatitis or epididymo-orchitis and four-week courses of doxycycline achieve equivalent responses to weekly doses of azithromycin for four weeks.[59,60]

In the guidelines for the treatment of PID from the Centers for Disease Control and Prevention, doxycycline combined with either ceftriaxone (+/– metronidazole), cefoxitin or cefotetan is recommended.[61] Because of concerns about poorer activity against *M. genitalium*, as well as greater ease of use with azithromycin, doxycycline is being used less commonly now in some locations.[62–64] In a comparison of the treatment of PID using ceftriaxone combined with either three doses of azithromycin over two weeks or two weeks of doxycycline, better results were obtained in the azithromycin group.[65]

Three-week courses of doxycycline can be used in the therapy of lymphogranuloma venereum and granuloma inguinale (donovanosis).[66,67] Due to high rates of resistance, doxycycline is no longer recommended for the treatment of gonorrhoea or chancroid (*Haemophilus ducreyi*) without confirming susceptibility first.[67,68]

Cell wall deficient bacteria such as Rickettsia

Worldwide, an increasing number of species of *Rickettsia* are being recognised that cause human disease. These are divided based on their clinical characteristics into the spotted fever group, the typhus group and the scrub typhus group. Doxycycline is the treatment of choice for these rickettsial infections, generally given as 100-mg twice daily for 5–7 days. It is also the treatment of choice in infections caused by *Ehrlichia* spp. and *Anaplasma phagocytophilum*.[69] In most cases, response is rapid, although there have been recent reports about delayed response or reduced susceptibility in cases of scrub typhus and murine typhus.[70]

Q fever infections can be self-limiting but if treatment is used in acute cases, doxycycline for 14 days is preferred. In cases of Q fever endocarditis, prolonged therapy is required, generally for 18 months and combined with another agent such as hydroxychloroquine.[71]

Bartonella

Bartonella spp. can cause a variety of manifestations, including trench fever, endocarditis, pyrexia of unknown origin, cat scratch disease, bacillary angiomatosis, peliosis hepatis and splenitis, Oroya fever, verruga peruana, retinitis and pericarditis. Doxycycline has activity against *Bartonella* spp. and is recommended as first-line therapy for many of these conditions, either alone, or in the more serious illnesses, combined with other agents such as gentamicin or rifampicin. It is generally given for prolonged periods.[72]

Spirochetal infections

In patients with early Lyme disease, treatment with doxycycline for 10–21 days is as effective as other agents such as amoxycillin or cefuroxime.[73,74] One potential benefit of choosing doxycycline is that it will also have activity against *Ehrlichia* or *Anaplasma* infections that may be also be transmitted by ticks. If dissemination has occurred, with either neurologic or cardiac complications, it can still be used and should be given for 21 days, with results equivalent to intravenous penicillin G or ceftriaxone.[75–77] In late cases of Lyme disease that are associated with arthritis, a month of doxycycline is effective in the majority.[78] Other *Borrelia* infections such as relapsing fever can also be treated with doxycycline, although there is greater experience using tetracycline and penicillins.

14–28 days of doxycycline is an alternative for treating primary or secondary syphilis in the penicillin-allergic patient.[79] It is also likely that this will be adequate treatment of endemic treponematoses such as yaws, although clinical studies about this are lacking.

FOI 26-3132 - Document 5

Doxycycline is effective therapy for leptospirosis and is equivalent to both penicillin and cefotaxime.[80] As rickettsial infections may be prevalent in some areas with leptospirosis and the two infections may occur together or be difficult to distinguish, doxycycline is the preferred option in such locations.

Potential agents of bioterrorism

Both naturally occurring anthrax and that due to bioterrorism can be treated with doxycycline. Naturally occurring cutaneous anthrax is treated for 5–7 days while both treatment and post-exposure prophylaxis in the event of bioterrorism require 60 days of therapy. Inhalational anthrax is generally more severe and combination therapy is preferred.[81,82]

Tularaemia is another potential bioterrorism agent. Recommended therapy is generally streptomycin or gentamicin, although doxycycline is a suitable alternative, especially if the patient is not seriously ill.[83] Similar recommendations are made for infections due to *Yersinia pestis*, with 7–10 days therapy suggested and high response rates seen.[84]

Malaria

For chemoprophylaxis against malaria in areas with chloroquine resistance, one of the most commonly used agents is doxycycline 100-mg daily taken from two days before entering until four weeks after leaving the malarial area. This offers protection of over 93% and is equivalent to mefloquine although the tolerability of and compliance with weekly mefloquine may be better.[85–87] At lower doses, or when taken for only two weeks after travel, breakthrough cases of malaria, particularly *Plasmodium vivax*, are not uncommon. In general, for travel to malaria-endemic areas with chloroquine resistance, options for prophylaxis are doxycycline, mefloquine and atovaquone-proguanil. The choice is based on cost, patient preference and ability to adhere, co-morbid illnesses, history of side effects, and presence of widespread resistance to any of these agents in the travel destinations. It is important to seek up to date advice on the rates of drug-resistant malaria in the countries that will be visited.

For treatment of malaria, particularly that caused by *P. falciparum*, doxycycline also has a role as adjunctive therapy. It can be combined with quinine (or quinidine), mefloquine, artesunate or artemether to improve rates of cure.

Miscellaneous uses

Doxycycline can be used as a second-line treatment option combined with other agents to treat *Helicobacter pylori*, particularly when clarithromycin resistance is present.[88,89] There is, however, greater experience with tetracycline for this indication. Ocular trachoma infections can also be treated with doxycycline although generally either oral azithromycin or topical tetracycline are preferred.[90] Following induction therapy for Whipple's disease with ceftriaxone or penicillin, doxycycline combined with hydroxychloroquine for one year can be used as maintenance therapy in patients unable to take co-trimoxazole.[91]

In patients with cholera, antibiotics are an adjunct to adequate fluid and electrolyte replacement but may have a role in reducing *Vibrio* excretion as well as volume of diarrhoea. Doxycycline (or tetracycline) has been the treatment of choice although resistance to this class is seen.[92,93] Infections due to non-cholera *Vibrio* spp. and *Aeromonas* spp., which generally involve the skin or gastro-intestinal tract but may cause septicaemic illnesses, can also be treated with doxycycline.[91,92]

In treating adults with brucellosis, doxycycline for 45 days combined with initial streptomycin is frequently given as first-line treatment. Substitution of gentamicin for the streptomycin leads to equivalent outcomes.[94] Alternatively, 45 days of doxycycline plus rifampicin is sometimes used and is superior to doxycycline plus a fluoroquinolone.[95] In patients with osteoarticular brucellosis, aminoglycoside-doxycycline therapy is preferred.[96] Triple antibiotic combination with doxycycline, rifampicin and an aminoglycoside appears to reduce the relapse rate.[95]

Actinomycosis is another infection that can be treated with doxycycline, although penicillins are usually first-line therapy.[97] For infections due to *Nocardia* spp., minocycline is preferred although doxycycline maintenance therapy may be used in patients who are intolerant of this and co-trimoxazole. Melioidosis is treated initially with IV carbapenems or ceftazidime but this is then followed up with at least three months of oral therapy, generally using co-trimoxazole plus doxycycline, for which the MICs are generally low.[98,99]

Rat bite fever due to infection with *Streptobacillus moniliformis* is generally treated with penicillins although doxycycline can be substituted in the allergic patient. Occasional isolates of rapid growing mycobacteria such as *M. fortuitum* are susceptible to doxycycline although this is rarely used clinically.[100] Prolonged courses of doxycycline over three to six months are sometimes used to treat patients with tropical sprue.[101]

An interesting newer use of doxycycline has been for *Wolbachia* endosymbiont infection in filarial infections such as onchocerciasis and Wuchererian filariasis. It is used for 6 weeks and by killing the symbiotic bacteria that assist in the life cycle of the filaria, interrupts embryogenesis in the adult worms for prolonged periods.[102–105]

Non-infectious conditions that can be treated with doxycycline

Doxycycline has also been used in a variety of non-infectious conditions. Most commonly, it can be used for severe acne, although part of its effect may be due to anti-bacterial activity against *Propionibacterium acnes*. Other examples of its use are in periodontitis, rosacea, bullous dermatoses, neutrophilic diseases, pyoderma gangrenosum, sarcoidosis, multiple sclerosis, the medical therapy of aortic aneurysms, autoimmune disorders such as rheumatoid arthritis and scleroderma, and as a sclerosant in pleurodesis procedures and for lymphatic or vascular malformations.[106–115]

An interesting potential use for doxycycline is its use in treating or preventing certain cancer metastases. This particularly relates to bone metastases from breast or prostate primaries, possibly due to the inhibition of certain MMPs that are mediators in the formation of this complication.[116] However, *in vitro* activity against a range of cancers has been demonstrated with members of the tetracycline family, including the recently developed molecule, Col-3.[7,116]

Safety

Doxycycline is generally well tolerated, especially compared with older tetracyclines and minocycline.[34,117]

Gastrointestinal

Common symptoms include nausea, vomiting, diarrhoea and epigastric burning. More severe reactions include oesophagitis, oesophageal ulceration, and mediastinitis. The risk of oesophageal ulceration can be reduced with the use of enteric-coated preparations, and with the monohydrate (rather than hydrochloride) formulation. Patients should take doxycycline with sufficient fluids and remain upright for 30 minutes after administration to reduce symptoms. Doxycycline should be avoided in patients with oesophageal compression or obstruction. Other gastrointestinal effects include *Clostridium difficile*-associated diarrhoea, although this occurs less frequently than with other antibiotics such as clindamycin.[118] Tetracycline-induced hepatotoxicity is increased in the presence of hepatic impairment, however the risk with doxycycline is lower or minimal compared with other tetracyclines.[119]

Dermatological and hypersensitivity

Doxycycline may cause photosensitivity and photo-onycholysis.[120] A wide variety of skin eruptions may occur, including erythematous, maculopapular and pustular rashes, pruritus, urticaria and fixed drug eruptions. Rarely hypersensitivity and serum sickness reactions,[121] Stevens-Johnson syndrome and toxic epidermal necrolysis may occur. The Jarisch-Herxheimer reaction may occur when doxycycline is used for treatment of spirochetal infections. Anaphylaxis has also been reported.

Bones and teeth

The accumulation of doxycycline in teeth and bones leads to discolouration of teeth and is more common in deciduous rather than permanent teeth. However if they are given to children whilst permanent teeth are still in development, this may lead to lifelong discolouration. Discolouration may also occur in permanent teeth, especially with concomitant poor dental hygiene and increased sunlight exposure.[122] It may also cause enamel dysplasia and bone deformities and impairment in bone growth.[123] Premature infants administered doxycycline demonstrated a 40% reduction in fibular bone growth that was reversible upon drug cessation.[124]

Neurological

Doxycycline has also been associated with benign intracranial hypertension. Symptoms usually improve upon cessation of doxycycline and concomitant administration of acetazolamide, however permanent

FOI 26-3132 - Document 5

visual acuity or visual field loss has occurred in a percentage of patients, necessitating optic nerve fenestration or CSF shunting.[125,126]

Other

Oropharyngeal and vaginal candidiasis from fungal overgrowth is common. Rare side effects include taste perversion, rhinitis, hiccups, and blood dyscrasias.[34,117,127] Drug-induced lupus is associated with minocycline but not doxycycline, however doxycycline may exacerbate systemic lupus erythematosus.[121] There are fewer side effects when doxycycline is used in lower doses without an increase in resistance of microorganisms.[34]

Pregnancy, lactation and children

Doxycycline can be given during the first 18 weeks of pregnancy but is contraindicated after this time due to potential discolouration and malformation of fetal teeth. It is potentially teratogenic in animal studies. There was a minor increase in fetal abnormalities (odds ratio 1.6, 95% confidence interval 1.1–2.3) in a large survey of over 18,000 pregnancies, however another study of 1795 doxycycline-exposed pregnancies demonstrated no increased risk of fetal abnormalities.[128,129] High doses of doxycycline have been associated with fatty necrosis of the liver in pregnant women, especially those with pyelonephritis. A short course of doxycycline for 7 to 10 days is considered safe during breast-feeding if there are no other alternatives available. Otherwise they should be used with caution as doxycycline is present in breast milk and may also lead to abnormalities in tooth development in the baby. Doxycycline is contraindicated in children younger than 8 years of age due to discolouration of teeth, enamel dysplasia and possible bone deformities. In rare situations doxycycline may be administered to children with a dose of 2.2 mg/kg twice daily where benefits of doxycycline outweigh the risks, such as infections with rickettsiae, *Yersinia pestis*, and *Burkholderia pseudomallei*.[130]

Efficacy

Resistance to doxycycline occurs due to acquisition of genes such as the *tet* and *otr* genes.[131] Generally they are acquired through conjugation from transposons, plasmids and integrons. The majority of these genes encode for efflux proteins that export tetracycline from the cell via an energy-dependent process. Some genes encode ribosomal protection proteins that result in ribosomal conformational changes, preventing doxycycline from binding to the ribosome. *Shigella dysenteriae* was the first tetracycline-resistant organism isolated. Since 1953 there has been a significant increase in doxycycline resistance in Gram-negative organisms including Enterobacteriaceae, Pseudomonodaceae, *Neisseria*, *Haemophilus, Mannheimia, Treponema, Vibrio* spp., and fastidious nonfermentative organisms such as *Kingella*, *Eikenella* and *Actinobacillus* spp.[1] Multi-drug resistance now occurs as *tet* genes are carried on integrons and plasmids with other genes encoding resistance to other antibiotics. Gram-positive organisms such as *Staphyloccocus aureus*, *Streptococcus agalactiae*, *Enteroccocus faecalis*, and *Streptococcus pneumoniae* have also developed doxycycline resistance and multi-drug resistance. Other organisms that may acquire *tet* and *otr* genes include *Mycoplasma*, *Ureaplasma*, *Actinomyces*, *Nocardia* and rapidly growing *Mycobacterium* species.

There are 2 newly discovered genes that encode tetracycline-inactivating enzymes in *Bacteroides* species.[132,133] Doxycycline resistance rarely occurs through chromosomal mutation where permeability of outer membrane pores affects diffusion of tetracycline into the cell. Clinically important chromosomal mutations occur in *H. pylori*, *M. avium* complex and *N. gonorrhoeae*. As yet there is no tetracycline resistance amongst obligate intracellular pathogens and protozoa.[134] Tetracycline-resistant *Chlamydophila suis* has been detected in pigs, so there is potential for doxycycline resistance in chlamydophilae.[135]

The increasing prevalence of doxycycline resistance in various geographic locations has limited its use as treatment for many organisms including Enterobacteriaceae, *S. aureus*, *S. pneumoniae* (including penicillin-resistant pneumococci) and *Bacteroides*.[136] Fortunately doxycycline remains active against a wide range of other infections where its use is now first line, for example, *Coxiella burnetii*, *Brucella melitensis*, *Borrelia burgdorferi*, and in penicillin-allergic patients with early syphilis.

Patient Preference

Doxycycline is inexpensive and available worldwide, and can be conveniently administered via oral or

FOI 26-3132 - Document 5

parenteral routes. This makes it practical for use in community and hospital settings. Excellent absorption and tissue distribution make it particularly useful for the severely ill patient compared with other antibiotics. Common adverse reactions such as gastrointestinal upset or oesophagitis can be prevented with careful attention to correct administration. Enteric-coated pellets have fewer gastrointestinal side effects.[34] Photosensitivity can be prevented or ameliorated with adequate attention to sun protection. It is important to remind women that the efficacy of the contraceptive pill is reduced and that barrier methods of contraception are required during and after doxycycline therapy.

Conclusions

Given its broad spectrum of activity and the wide array of clinical uses, including against some more obscure and difficult to diagnose conditions, doxycycline is sometimes referred to as the "secret weapon of the infectious diseases physician." Although the long acting macrolide azithromycin has taken on many of its uses, it retains a very important place in the antibiotic armamentarium. It is generally well tolerated and has excellent clinical activity in many sexually transmitted infections, rickettsial and related infections, Lyme disease, brucellosis, anthrax, Q fever and atypical respiratory pathogens as well as the prevention of malaria.

Disclosure

The authors report no conflicts of interest.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Journal of Clinical Epidemiology 144 (2022) 43–55

Journal of Clinical Epidemiology

ORIGINAL ARTICLE

Bias as a source of inconsistency in ivermectin trials for COVID-19: A systematic review. Ivermectin's suggested benefits are mainly based on potentially biased results

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Accepted 12 December 2021; Available online 18 December 2021

Abstract

Objective: The objective of this systematic review is to summarize the effects of ivermectin for the prevention and treatment of patients with COVID-19 and to assess inconsistencies in results from individual studies with focus on risk of bias due to methodological limitations.

Methods: We searched the L.OVE platform through July 6, 2021 and included randomized trials (RCTs) comparing ivermectin to standard or other active treatments. We conducted random-effects pairwise meta-analysis, assessed the certainty of evidence using the GRADE approach and performed sensitivity analysis excluding trials with risk of bias.

Results: We included 29 RCTs which enrolled 5592 cases. Overall, the certainty of the evidence was very low to low suggesting that ivermectin may result in important benefits. However, after excluding trials classified as "high risk" or "some concerns" in the risk of bias assessment, most estimates of effect changed substantially: Compared to standard of care, low certainty evidence suggests that ivermectin may not reduce mortality (RD 7 fewer per 1000) nor mechanical ventilation (RD 6 more per 1000), and moderate certainty evidence shows that it probably does not increase symptom resolution or improvement (RD 14 more per 1000) nor viral clearance (RD 12 fewer per 1000).

Conclusion: Ivermectin may not improve clinically important outcomes in patients with COVID-19 and its effects as a prophylactic intervention in exposed individuals are uncertain. Previous reports concluding important benefits associated with ivermectin are based on potentially biased results reported by studies with substantial methodological limitations. Further research is needed. © 2022 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Keywords: COVID-19; SARS-CoV-2; Coronavirus Infections; Systematic review; ivermectin; bias

Conflict of interest: The authors have declared that no competing interests exist. Authors hold sole responsibility for the views expressed in the manuscript, which may not necessarily reflect the opinion or policy of the Pan American Health Organization.

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https://doi.org/10.1016/j.jclinepi.2021.12.018
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FOI 26-3132 - Document 5

What is new?

- We found substantial differences in the results of studies with or without important methodological limitations.
- Ivermectin's suggested benefits are mainly based on potentially biased results.
- There is substantial uncertainty on ivermectin's effects for patients with COVID-19 or exposed to SARS-COV-2 and further research is needed.

1. Introduction

There is an urgent need to expand the evidence base on interventions for the prevention and treatment of COVID-19, an infection caused by SARS-CoV-2 that has the potential of progression into pneumonia, multi-organ failure and death [1]. The COVID-19 pandemic has seen a rapid increase in the number of studies testing potential therapeutic options, raising concerns about the quality and lack of scientific integrity, and also about the spread of this information, leading to the so-called "infodemic" [2,3]. According to the World Health Organization (WHO) international registry of clinical trials platform (ICTRP) [4], hundreds of potential interventions are being assessed in more than 10,000 clinical trials and observational studies.

Many drugs including ivermectin, were repurposed for the treatment of COVID-19, most often based on biological plausibility, *in vitro* research, or pathophysiological considerations. Ivermectin is a successful broad-spectrum antiparasitic, included in WHO essential medicines list used to treat several neglected tropical diseases [5]. It emerged as a potential treatment for COVID-19 in mid-2020, following an *in vitro* study demonstrating its anti-viral properties [6].

Multiple systematic reviews have assessed the benefits and harm of ivermectin for COVID-19 patients with inconsistent findings and conclusions [7]. Although some organizations and groups have argued strongly in favor of implementing ivermectin for treatment and/or prevention of COVID-19 [8], current key clinical practice guidelines recommend against its use outside the context of clinical trials [9–12].

Reasons for these major discrepancies are probably related to different evidence analytical and/or interpretation approaches. Assessing the risk of bias is one of the pillars of any systematic review and has proven to be essential for evidence interpretation in the present pandemic context where results of studies with major methodological limitations have led to erroneous conclusions, waste of resources and patients' exposure to potentially harmful interventions [3,13,14]. Nevertheless, most available systematic reviews on ivermectin for COVID-19 have not appropriately assessed risk of bias as a potential explanation for inconsistency between trial results. Therefore, this systematic review aims to summarize the best available evidence on ivermectin for prevention and treatment of COVID-19 patients and explore potential explanations for heterogeneity in RCTs results with focus on studies methodological limitations.

2. Methods

This systematic review report is consistent with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [15].

2.1. Protocol registration

This systematic review is part of a larger project that aims to conduct multiple systematic reviews for different questions relevant to COVID-19. The protocol stating the shared objectives and methodology of these reviews was published elsewhere [16].

2.2. Search strategy

We systematically searched in the Living OVerview of Evidence (L.OVE; https://app.iloveevidence.com/covid19) platform, for studies on Ivermectin for COVID-19. L.OVE platform is a system that maps PICO (Patient–Intervention–Comparison–Outcome) questions to a repository developed by Epistemonikos Foundation and is the search platform for the Pan American Health Organization (PAHO) living systematic review of potential therapeutics for COVID-19.[7] The search terms and databases covered are described on the L.OVE platform methods section available at: https://app.iloveevidence.com/covid19/methods. The repository that feeds the L.OVE platform was developed and is maintained through the automated and manual screening of multiple databases, trial registries, preprint servers and other sources. The last version of the methods, the total number of sources screened, and a living flow diagram and report of the project is updated regularly on the website. The searches cover the period from inception date of each database. We last searched the platform on July 6, 2021. There were no restrictions applied to the language or publication status.

2.3. Study selection

Two reviewers (A.I and G.R) working independently and in duplicate, performed study selection, including screening of titles and abstracts and of potentially eligible full-text articles. Reviewers resolved disagreements by discussion.

We included randomized controlled trials (RCTs) that recruited adults with suspected, probable, or confirmed

FOI 26-3132 - Document 5

COVID-19, or that were exposed to SARS-COV-2, comparing systemic ivermectin alone or in combination with other drugs, against placebo, standard care or other interventions, and reported on clinical important outcomes (see "Outcomes of interest" below). We included trials regardless of publication status (peer reviewed, in press, or preprint) or language. No restrictions were applied based on severity of COVID-19 illness, setting in which the trial was conducted (e.g., outpatient, inpatient, critical), dose administered or duration of treatment. We excluded studies in which inhaled ivermectin was used as intervention.

2.4. Data extraction

For each eligible trial one reviewer (A.I) extracted data using a standardized, pilot-tested data extraction form. The reviewer collected information on trial characteristics (trial registration, publication status, study status, design), participant characteristics (country, age, sex, comorbidities, and severity), and outcomes of interest. Extracted data was confirmed by a second reviewer (F.T). Discrepancies were resolved through discussion

2.5. Outcomes of interest

We selected clinically important outcomes considering published prioritization exercises performed in the context of different clinical practice guidelines [9,11]. We included all-cause mortality and invasive mechanical ventilation as critical outcomes, and symptom resolution or improvement, hospitalizations, viral clearance, symptomatic infection, and severe adverse events as important outcomes. For symptom resolution or improvement, we considered the proportion of patients with complete resolution of symptoms, or the proportion of patients discharged from hospital or the proportion of patients with important symptom improvement as reported by investigators. For viral clearance we considered the proportion of patients with negative PCR test. For severe adverse events we used the definition implemented by the investigators.

2.6. Risk of Bias

Two reviewers (A.I and M.R) independently assessed the risk of bias of all included trials using the revised Cochrane Risk of Bias 2.0 tool for randomized trials (RoB 2) [17], focusing on randomization, allocation concealment, blinding, attrition, or other biases relevant to the estimates of effect. In assessing the domain "risk of bias arising from randomization process", in addition to exploring the balance of baseline prognostic in individual trials, we assessed overall balance by constructing Forest plots. We assumed that lack of blinding was less likely to introduce bias to "mortality" and "mechanical ventilation" outcomes hence we assessed risk of bias separately for those two outcomes as follows. For "mortality" and "mechanical ventilation" outcomes we rated trials at high risk of bias overall if one or more domains were rated as "high risk of bias", and as "some concerns" if no domains were rated as "high risk of bias" and "Risk of bias arising from randomization process" and/or "Risk of bias due to missing outcome data" and/or "Risk of bias in selection of reported results" domains were classified as "some concerns". The remaining trials were rated as "low risk of bias". For other outcomes, we rated trials at high risk of bias overall if one or more domains were rated as "high risk of bias", as "some concerns" if no domains were rated as "high risk of bias" and one or more domains were rated as "some concerns", and low risk of bias overall if all domains were rated as "low risk of bias". Reviewers resolved discrepancies by discussion.

2.7. Data synthesis

We summarized the effect of interventions on selected outcomes using relative risks (RRs) and corresponding 95% confidence intervals (95%CIs). We conducted frequentist random-effects pairwise meta-analyses using the R package "meta" in RStudio Version 1.4.1103 [18]. For the primary analysis, we assumed that interventions used in some trials as active comparators (hydroxychloroquine and lopinavir-ritonavir), are not related to important effects in patients with COVID-19 [9]. We considered those interventions as standard of care and performed sensitivity analysis to assess the robustness of results (see subgroup and sensitivity analyses).

2.8. Certainty of the evidence

We assessed the certainty of evidence using the grading of recommendations assessment, development, and evaluation (GRADE) approach [19]. Two methodologists with experience in using GRADE rated each domain for each comparison separately and resolved discrepancies by consensus. We rated the certainty for each comparison and outcome as high, moderate, low, or very low, based on considerations of risk of bias, inconsistency, indirectness, publication bias, and imprecision. We made judgments of imprecision using a minimally contextualised approach with the null effect as a threshold. This minimally contextualised approach considers whether the 95%CI includes the null effect, or, when the point estimate is close to the null effect, whether the 95%CI lies within the boundaries of small but important benefit and harm that corresponds to every outcome assessed [20,21]. To define severe or very severe imprecision we considered if the 95%CI included not only the null effect, but important benefits and harms. We used MAGIC authoring and publication platform (https://app.magicapp.org/) to generate the tables summarizing our findings. We calculated the absolute risks and risk differences from the RRs (and their 95%CIs) and

FOI 26-3132 - Document 5

the median risk in the control groups of studies reporting on severe patients for "mortality" and "mechanical ventilation" outcomes. For the remaining outcomes we used RRs (and their 95%CIs) and the median risk in the control groups of all analysed trials.

To communicate our findings and conclusions using statements we followed published guidance [22].

2.9. Subgroup and sensitivity analyses

To assess if overall estimates of effects could be influenced by trials reporting potentially biased results, we performed sensitivity analysis excluding trials categorized as "high risk of bias" and "some concerns". We expected smaller effects after excluding those trials. In addition, as there is high certainty evidence on the lack of efficacy of some interventions for the treatment of patients with COVID-19 such as hydroxychloroquine and Lopinavir-Ritonavir [9], for the primary analysis, we considered those interventions as a part of the standard of care. However, we performed sensitivity analyses excluding trials in which hydroxychloroquine or Lopinavir-Ritonavir were used as comparators. We performed subgroup analysis based on intervention implemented and baseline disease severity, we expected larger effects in trials in which ivermectin was implemented in combination with other interventions and in patients with less severe disease.

2.10. Update of this systematic review

An artificial intelligence algorithm deployed in the COVID-19 topic of the L.OVE platform (https://app.iloveevidence.com/covid19) will provide instant notification of articles with a high likelihood of eligibility. These will be screened by paired reviewers iteratively who will also conduct data extraction and updates of the estimates of effects and certainty of the evidence. We will consider resubmission to a journal if there is a substantial modification of the effect estimate or certainty of the evidence for ivermectin, at the discretion of the reviewer team.

3. Results

The search strategy identified 680 potentially eligible records, of which 29 RCTs (reported in 78 references) were included. We identified two additional studies which we decided not to include. One was reported as a cluster randomized trial but methods and results were poorly reported and not consistent with a RCT [23]. The other was mentioned in a published review[24] but we were unable to obtain the full text [25]. We intended to contact the authors of these and other three included studies[26, 27, 28] for further methodological details by email, but only one responded [28]. On July 14, 2021, one of the included studies was retracted from the preprint server due to research misconduct concerns that are being investigated [29]. As the primary aim of our review was to assess the influence of potentially biased results on ivermectin's effects interpretation, we decided not to exclude it. The selection process is described by the PRISMA flow diagram in S1 Figure. The list of excluded studies is available upon request.

3.1. Trial characteristics

There was a total of 5592 patients from 29 RCTs, [26–54] in which ivermectin was compared against standard of care or other treatments (S1 Table). Twenty trials were published in peer reviewed journals and nine were only published as preprints. One trial reported the results of three different cohorts, one of severe patients, one of mild patients and one of exposed persons, we therefore analyzed each cohort as a different trial [29]. The sample size ranged from 24 to 1342, with 2830 assigned to Ivermectin and 2483 assigned to control. Eighteen trials included patients with mild to moderate COVID-19 [26,28-30,32-45], three studies included patients with severe to critical COVID-19 [29,46,47], six studies included patients with mild to critical disease [27,48,49,50,51,52], and four studies included non-infected patients exposed to SARS-COV-2 [29,31,53,54].

Ivermectin administered dose varied from 12 mg once to 400 μgm/kg once a day for 4 days. Ivermectin alone was used in most trials but five in which the intervention implemented was a combination of ivermectin with doxycycline [26,32,34,48], or iota-Carrageenan [54]. Comparator was standard of care with or without placebo in most trials. Active comparators included hydroxychloroquine or cloroquine [29,47], hydroxychloroquine plus azythomicin [32], lopinavir-ritnonavir[50] and vitamin C [31].

3.2. Risk of bias

The risk of bias assessment of the 29 included trials is summarized in (Table 1). For mortality and mechanical ventilation, our assessment resulted in high risk of bias for four RCT (including the study retracted due to misconduct concerns) [29], some concerns for two and low risk of bias for seven RCTs. For all remaining outcomes, our assessment resulted in high risk of bias for thirteen RCTs, some concerns for nine and low risk of bias for seven RCTs. Most trials did not provide enough information to assess baseline differences between arms. Overall assessment of baseline prognostic factors suggested that ischemic heart disease was less frequent in patients assigned to ivermectin (S2 Figure). A detailed description of the trials' methodological limitations is provided in a supplementary table (S2 Table).

FOI 26-3132 - Document 5

Table 1. Risk of bias of included trials

Study	Risk-of-bias arising from randomization process	Risk-of-bias due to deviations from the intended interventions	Risk-of-bias due to misssing outcome data	Risk-of-bias in measurement of the outcome	Risk-of-bias in selection of the reported result	Overall Risk-of-bias judgement	
						Mortality and Invasive mechanical ventilation	Symptom resolution or improvement, hospitalization, infection, viral cleareance and adverse events
Shouman et al. [53]	High	Some Concerns	Low	Some Concerns	Low	-	High
Chowdhury et al. [32]	High	Some Concerns	Low	Some Concerns	Low	-	High
Podder et al. [33]	High	Some Concerns	Low	Some Concerns	Low	-	High
Hashim et al. [48]	High	Some Concerns	Low	Some Concerns	Low	High	High
Elgazzar et al. [29]	High	Some Concerns	Low	Some Concerns	Low	High	High
Krolewiecki et al. [35]	Low	Some Concerns	Low	Some Concerns	Low	Low	Some concerns
Niaee et al. [49]	High	Some Concerns	Low	Some Concerns	Low	High	High
Ahmed et al. [26]	High	Low	Low	Low	Some concerns	-	High
Chaccour et al. [30]	Low	Low	Low	Low	Low	-	Low
Chachar et al. [36]	Some Concerns	Some Concerns	Low	Some Concerns	Low	-	Some concerns
Babalola et al. [50]	Low	Some Concerns	Low	Some Concerns	Low	-	Some concerns
Kirti et al. [37]	Low	Low	Low	Low	Low	Low	Low
Chahla et al. [54]	High	Some Concerns	Low	Some Concerns	Low	-	High
Mohan et al. [38]	Low	Low	Low	Low	Low	-	Low
Shahbaznejad et al. [51]	Low	Low	Low	Low	Low	Low	Low
Samaha et al. [39]	High	Some Concerns	Low	Some Concerns	Low	-	High
Bukhari et al. [40]	High	Some Concerns	Low	Some Concerns	Low	-	High
Okumus et al. [46]	High	Some Concerns	Low	Some Concerns	Low	High	High
Beltran et al. [27]	Some Concerns	Low	Low	Low	Low	Some concerns	Some concerns
López-Medina et al. [41]	Low	Low	Low	Low	Low	Low	Low
Bermejo Galan et al. [47]	Low	Low	Low	Low	Low	Low	Low
Pott-Junior et al. [52]	Low	Some Concerns	Low	Some Concerns	Low	-	Some concerns
Kishoria et al. [42]	Low	Some Concerns	Low	Some Concerns	Low	-	Some concerns
Seet et al. [31]	Low	Some Concerns	Low	Some Concerns	Low	-	High
Mahmud et al. [33]	Low	Low	Some Concerns	Low	Low	Some concerns	Some concerns
Abd-Elsalam et al. [43]	Low	Some Concerns	Low	Some Concerns	Low	Low	Some concerns
Biber et al. [44]	Some Concerns	Low	Some Concerns	Low	Low	-	Some concerns
Faisal et al. [45]	High	Some Concerns	Low	Some Concerns	Low	-	High
Vallejos et al. [28]	Low	Low	Low	Low	Low	Low	Low

FOI 26-3132 - Document 5

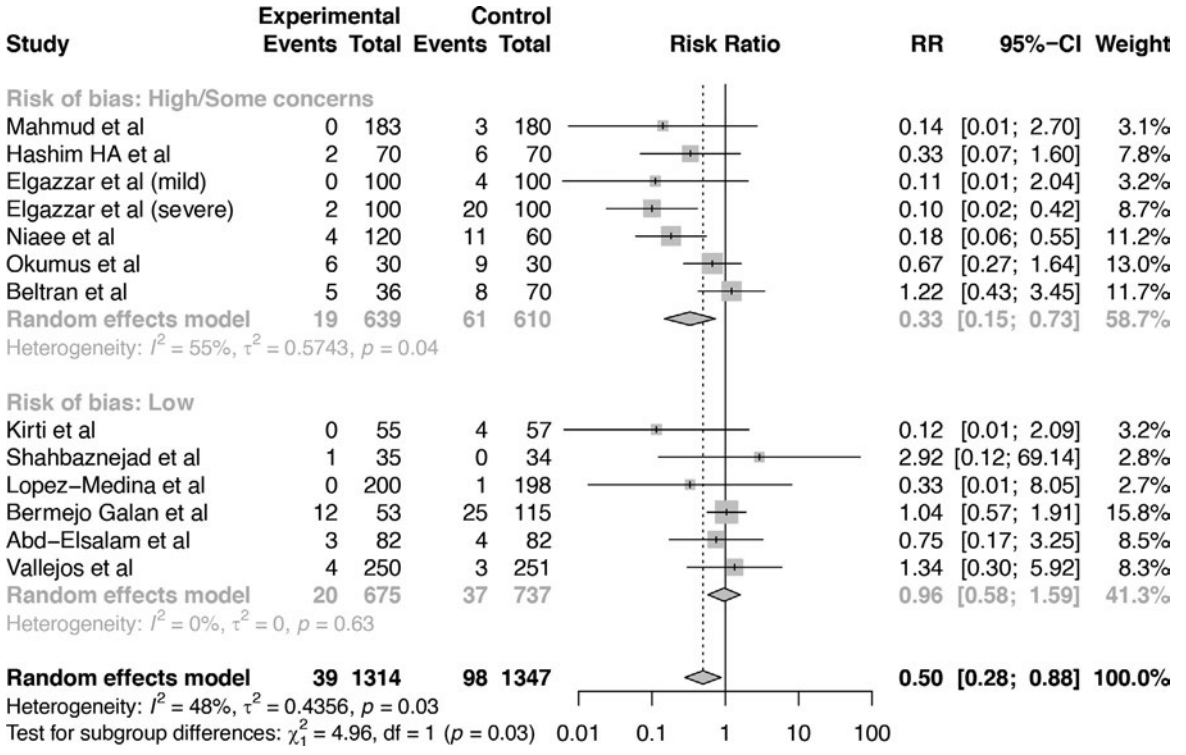


Fig. 1. Results of primary analysis and sensitivity analysis excluding trials with significant methodological limitations.

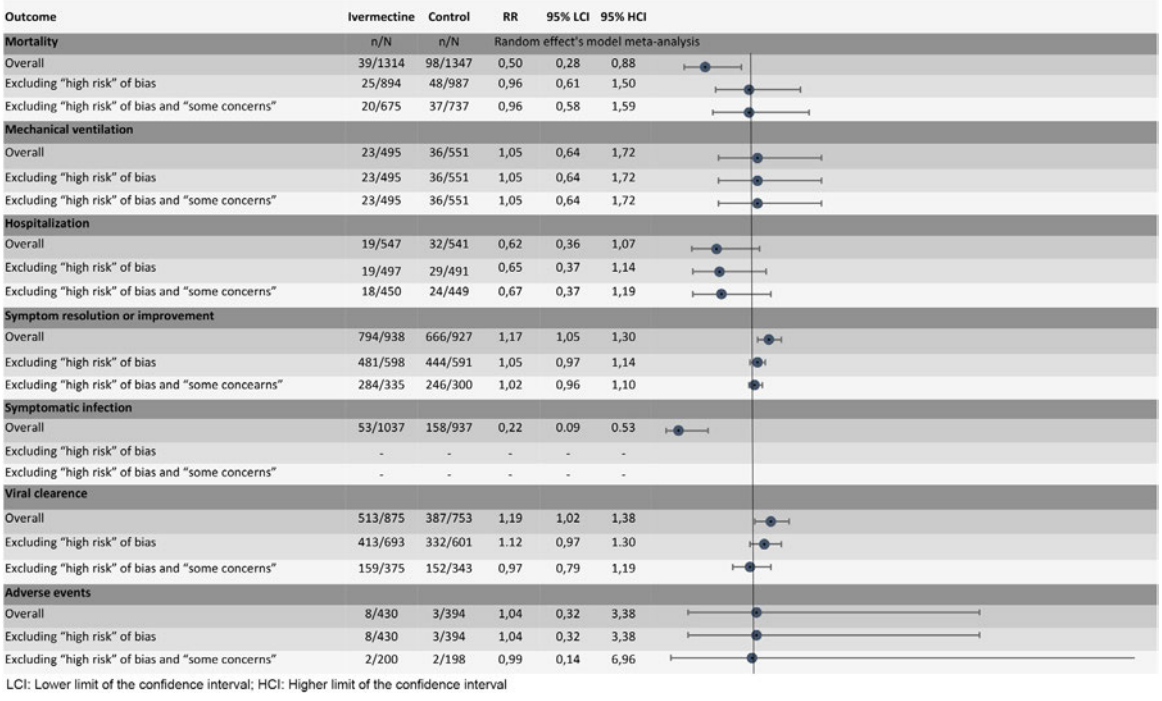


Fig. 2. Comparison: ivermectin vs. Standard of care; Outcome: mortality; Analysis: subgroups by risk of bias classification.

3.3. Effects on assessed outcomes

Table 2. and Fig. 1. provide a summary of finding for all assessed outcomes.

3.4. Mortality

Twelve trials with 2661 patients reported on mortality [27,28,29,34,37,41,43,46-49,51]. Ivermectin may reduce mortality (RR 0.50, 95% CI:0.28 to 0.88; RD 91

FOI 26-3132 - Document 5

Table 2. Summary of findings table

Outcome Timeframe	Study results and measurements	Absolute effect estimates		Certainty of the Evidence (Quality of evidence)	Plain text summary
		SOC	Ivermectin		
Mortality (Overall)	Relative risk: 0.50 (CI 95% 0.28 – 0.88) Based on data from 2661 patients in 12 studies Follow up: median 30 days	183 per 1000 Difference: 91 fewer per 1000 (CI 95% 132 fewer – 22 fewer)	92 per 1000	Low Due to serious risk of bias and serious inconsistency[a,b]	Ivermectin may reduce mortality
Mortality (excluding "some concerns" and "high risk of bias" trials)	Relative risk: 0.96 (CI 95% 0.58 – 1.59) Based on data from 1412 patients in 6 studies Follow up: median 25.5 days	183 per 1000 Difference: 7 fewer per 1000 (CI 95% 71 fewer – 92 more)	176 per 1000	Low Due to very serious imprecision[c]	Ivermectin may have little or no difference on mortality
Mechanical ventilation (overall - all studies classified as low risk of bias)	Relative risk: 1.05 (CI 95% 0.64 – 1.72) Based on data from 1046 patients in 6 studies Follow up: median 30 days	119 per 1000 Difference: 6 more per 1000 (CI 95% 43 fewer – 86 more)	125 per 1000	Low Due to very serious imprecision[c]	Ivermectin may have little or no difference on mechanical ventilation
Symptom resolution or improvement (overall)	Relative risk: 1.17 (CI 95% 1.05 – 1.3) Based on data from 1865 patients in 11 studies Follow up: median 10 days	714 per 1000 Difference: 121 more per 1000 (CI 95% 36 more – 214 more)	835 per 1000	Low Due to serious risk of bias and serious inconsistency[a,b]	Ivermectin may increase symptom resolution or improvement
Symptom resolution or improvement (excluding "some concerns" and "high risk of bias" trials)	Relative risk: 1.02 (CI 95% 0.96 – 1.1) Based on data from 635 patients in 3 studies Follow up: median 14 days	714 per 1000 Difference: 14 more per 1000 (CI 95% 29 fewer – 71 more)	728 per 1000	Moderate Due to serious imprecision[d]	Ivermectin probably has little or no difference on symptom resolution or improvement
Hospitalization (overall)	Relative risk: 0.62 (CI 95% 0.36 – 1.07) Based on data from 1088 patients in 4 studies Follow up: median 17.5 days	54 per 1000 Difference: 21 fewer per 1000 (CI 95% 35 fewer – 4 more)	35 per 1000	Low Due to very serious imprecision[e]	Ivermectin may reduce hospitalizations

(*continued on next page*)

Table 2 (*continued*)

Outcome Timeframe	Study results and measurements	Absolute effect estimates		Certainty of the Evidence (Quality of evidence)	Plain text summary
		SOC	Ivermectin		
Hospitalization (excluding some concerns and high risk of bias studies)	Relative risk: 0.67 (CI 95% 0.37 – 1.19) Based on data from 899 patients in 2 study Follow up: median 25.5 days	54 per 1000 Difference: 18 fewer per 1000 (CI 95% 34 fewer – 8 more)	36 per 1000	Low Due to very serious imprecision[e]	Ivermectin may reduce hospitalizations
Symptomatic infection (overall - all studies classified as high risk of bias)[14]	Relative risk: 0.22 (CI 95% 0.09 – 0.53) Based on data from 1974 patients in 4 studies Follow up: median 21 days	159 per 1000 Difference: 124 fewer per 1000 (CI 95% 145 fewer – 75 fewer)	35 per 1000	Low Due to very serious risk of bias[a]	Ivermectin may decrease symptomatic infection
Viral clearance (overall)	Relative risk: 1.19 (CI 95% 1.02 – 1.38) Based on data from 1628 patients in 13 studies Follow up: median 6 days	400 per 1000 Difference: 76 more per 1000 (CI 95% 8 more – 152 more)	476 per 1000	Low Due to serious risk of bias and serious inconsistency[a,b]	Ivermectin may increase viral clearance
Viral clearance (excluding "some concerns" and "high risk of bias" trials)	Relative risk: 0.97 (CI 95% 0.79 v 1.19) Based on data from 718 patients in 3 studies Follow up: median 5 days	400 per 1000 Difference: 12 fewer per 1000 (CI 95% 84 fewer – 76 more)	388 per 1000	Moderate Due to very serious imprecision[d]	Ivermectin probably has little or no difference on viral clearance
Severe adverse events (overall)	Relative risk: 1.04 (CI 95% 0.32 – 3.38) Based on data from 824 patients in 4 studies Follow up: median 29 days	5 per 1000 Difference: 0 fewer per 1000 (CI 95% 3 fewer – 12 more)	5 per 1000	Very low Due to serious risk of bias and very serious imprecision[a,c]	We are uncertain whether ivermectin increases or decreases severe adverse events
Severe adverse events (excluding "some concerns" and "high risk of bias" trials)	Relative risk: 0.99 (CI 95% 0.14 – 6.96) Based on data from 398 patients in 1 study Follow up: median 21 days	5 per 1000 Difference: 0 fewer per 1000 (CI 95% 4 fewer – 30 more)	5 per 1000	Very low Due to extremely serious imprecision[f]	We are uncertain whether ivermectin increases or decreases severe adverse events

[a] Risk of Bias: Serious or very serious. See Table 1 and Appendix Table 2.
[b] Inconsistency: Serious. The confidence interval of some of the studies do not overlap with those of most included studies.
[c] Imprecision: Very serious. 95%CI includes important benefits and harms.
[d] Imprecision: Serious. 95%CI includes important benefits.
[e] Imprecision: Very serious. 95%CI includes absence of benefits and low number of events.
[f] Imprecision: Extremely serious. 95%CI includes important benefits and harms and very low number of events.

FOI 26-3132 - Document 5

fewer per 1,000 participants, 95% CI: 132 fewer to 22 fewer). The certainty of the evidence was low because of serious risk of bias and serious inconsistency (I^2 48%). Sensitivity analysis excluding six trials classified as "some concerns" or "high risk of bias" showed that ivermectin may not reduce mortality (RR 0.96, 95% CI: 0.58 to 1.59; RD 7 fewer per 1,000 participants, 95% CI: 71 fewer to 92 more) (Fig. 2).

3.5. Mechanical ventilation

Six trials with 1046 patients, classified as "low risk of bias", reported on mechanical ventilation [28,35,37,43,47,51]. Ivermectin may not reduce the requirement of mechanical ventilation (RR 1.05, 95% CI:0.64 to 1.72; RD 6 more per 1,000 participants, 95% CI: 43 fewer to 86 more) (S3 Figure in S1 Appendix). The certainty of the evidence was low because of very serious imprecision.

3.6. Symptom resolution or improvement

Eleven trials with 1865 patients reported on symptom resolution or improvement [27,29,32,34,36-38,41,42,45,46]. Ivermectin may increase symptom resolution or improvement (RR 1.17, 95% CI:1.05 to 1.30); RD 121 more per 1,000 participants, 95% CI: 36 more to 214 more). The certainty of the evidence was low because of serious risk of bias and serious inconsistency (I^2 79%). Sensitivity analysis excluding eight trials classified as "some concerns" or "high risk of bias" showed that ivermectin probably does not increase symptom resolution or improvement (RR 1.02, 95% CI:0.96 to 1.10; RD 14 more per 1,000 participants, 95% CI: 29 fewer to 71 more) (S4 Figure in S1 Appendix).

3.7. Hospitalization

Four trials with 1088 patients reported on hospitalization[28,39,41,44]. Ivermectin may decrease hospitalizations (RR 0.62, 95% CI:0.36 to 1.07); RD 21 fewer per 1,000 participants, 95% CI: 35 fewer to 4 more). The certainty of the evidence was low because of very serious imprecision. Sensitivity analysis excluding two trials classified as "some concerns" or "high risk of bias" showed that ivermectin may decrease hospitalizations (RR 0.67, 95% CI:0.37 to 1.19); RD 18 fewer per 1,000 participants, 95% CI: 34 fewer to 10 more) (S5 Figure in S1 Appendix).

3.8. Symptomatic infection in exposed persons

Four trials including 1974 patients, classified as "high risk of bias", reported on symptomatic infection [29,31,53,54]. Ivermectin may reduce symptomatic infection (RR 0.22, 95% CI:0.09 to 0.53); RD 124 fewer per 1,000 participants, 95% CI: 145 fewer to 75 fewer) (S6 Figure in S1 Appendix). The certainty of the evidence was low because of very serious risk of bias.

3.9. Viral clearance

Thirteen trials with 1628 patients reported on viral clearance [26,28,32,33,34,37,38,40,42,44,46,50,52]. Ivermectin may increase viral clearance (RR 1.19, 95% CI: 1.02 to 1.38); RD 76 more per 1,000 participants, 95% CI: 8 more to 152 more). The certainty of the evidence was low because of serious risk of bias and serious inconsistency (I^2 56%). Sensitivity analysis excluding ten trials classified as "some concerns" or "high risk of bias" showed that ivermectin probably does not increase viral clearance (RR 0.97, 95% CI: 0.79 to 1.19); RD 12 fewer per 1,000 participants, 95% CI: 84 fewer to 76 more) (S7 Figure in S1 Appendix).

3.10. Severe adverse events

Four trials with 824 patients reported on severe adverse events [34,35,41,52]. It is uncertain if Ivermectin increases or decreases severe adverse events (RR 1.04, 95% CI: 0.32 to 3.38); RD 0 more per 1,000 participants, 95% CI: 3 fewer to 12 more). The certainty of the evidence was very low because of serious risk of bias and very serious imprecision. Sensitivity analysis excluding ten trials classified as "some concerns" or "high risk of bias" showed that it is uncertain if ivermectin increases or decreases severe adverse events (RR 0.99, 95% CI: 0.14 to 6.96); RD 0 more per 1,000 participants, 95% CI: 4 fewer to 30 more) (S8 Figure in S1 Appendix).

3.11. Additional analysis

Subgroup and sensitivity analysis did not suggest differential effects according to baseline disease severity, or when ivermectin was administered in combination with other interventions, or when it was compared against hydroxychloroquine or lopinavir-ritonavir, or when different outcome measurements time frames were used (S9 to S30 Figures in S2 Appendix). Visual inspection of the funnel plot for mortality suggested possible publication bias, (S31 Figure in S2 Appendix) however egger's test was not statistically significant ($P = 0.13$). Visual inspection of funnel plots for symptom resolution or improvement and viral clearance did not suggest publication bias, egger's test results $P = 0.48$ and $P = 0.25$ respectively (S32 and S33 Figures in S2 Appendix).

4. Discussion

This systematic review and meta-analysis provide a comprehensive overview of the available evidence on ivermectin for prevention and treatment of COVID-19. Overall, the body of evidence suggests that ivermectin may reduce mortality, may increase symptom resolution or improvement, may decrease hospitalizations, may increase viral clearance, and may decrease symptomatic infection

FOI 26-3132 - Document 5

in exposed individuals. However most trials have serious methodological limitations including lack of allocation concealment and lack of blinding, and reported results varied remarkably from striking benefits to null effects. GRADE assessment resulted in low or very low certainty of the evidence for all the outcomes, due to risk of bias, inconsistency, and imprecision. Visual inspection of funnel plot constructed for mortality outcome suggest possible publication bias which rises additional concerns about the certainty of the evidence on ivermectin's effects.

After excluding trials with significant methodological limitations inconsistency disappeared and results changed substantially. We found low certainty, due to imprecision, that ivermectin may not reduce mortality, nor reduce invasive mechanical ventilation, and moderate certainty evidence that ivermectin probably does not increase viral clearance or symptom resolution or improvement. Regarding hospitalizations, results did not change importantly suggesting that ivermectin may modestly reduce hospitalizations. However, certainty of the evidence remained low due to very serious imprecision. It is uncertain if ivermectin reduces or increases symptomatic infections in exposed individuals or increases severe adverse events as no trials classified as "low risk of bias" were identified, or the certainty of the evidence was very low.

Our systematic review has several strengths. The search strategy was comprehensive with explicit eligibility criteria, and no restrictions on language or publication status. We used a validated tool for risk of bias assessment and performed a thorough assessment providing details of trial limitations and potential important imbalances in baseline participant characteristics. We assessed the certainty of the evidence using the GRADE approach and interpreted the results considering absolute rather than relative effects.

Reporting was poor for a substantial number of included trials. For risk of bias assessment, we adopted a conservative approach and rated as low risk of bias only those trials for which it was clearly reported that no significant methodological limitations existed. Hence, we may have inappropriately classified some well executed trials as "some concerns" or "high risk of bias" due to their suboptimal reporting methods. Although for some trials we intended to contact the authors for clarification, most did not answer.

Multiple systematic reviews assessed ivermectin for COVID-19 [7]. Most of these reviews were already outdated at the time of writing this manuscript [55]. We did not identify studies included in other reviews that were not captured in our search strategy. Only five reviews incorporated a substantial proportion of the studies assessed in our review including a recently published systematic review by the Cochrane collaboration in which the authors excluded studies with high risk of bias or that compared ivermectin against other active interventions [24,56-59]. In agreement with our findings, all these reviews concluded that most of the studies assessing ivermectin for COVID-19 have considerable methodological limitations, and three judged the certainty of the evidence as low to very low for all outcomes [56,57] or not robust enough to justify ivermectin's use [58]. The authors of one systematic review concluded that ivermectin "may have a role in decreasing mortality in mildly/moderately ill COVID-19 patients" although they graded the certainty on ivermectin's effect on mortality as very low [59]. Bryant el at. graded the certainty of the evidence as low or very low for all outcomes except mortality for which they report moderate certainty in important mortality reduction. In contrast to our analysis, they reached this conclusion by not downgrading the certainty of the evidence for inconsistency even though they reported there was significant, not fully explained, heterogeneity in studies' results. In addition, for mortality outcome, they report a sensitivity analysis excluding high risk of bias studies which, in contrast to our findings, did not result in different estimates of effect from the primary analysis. This can be explained by the fact that the authors did not exclude a relevant number of studies with important methodological limitations, that they classified as "unclear" risk of bias [24].

Due to the excessive amount of rapidly published research on COVID-19, often referred to as an "infodemic" [2,3], the scientific community has already faced a similar scenario to the one described for ivermectin in the present review. Small studies with significant methodological limitations suggested benefits for steroids, lopinavir-ritonavir, interferon β-1a and convalescent plasma among others [60–63]. However, those potential benefits were seldom confirmed and mostly discarded by well-designed adequately powered studies [64–67]. The limitations in the body of evidence on ivermectin for COVID-19 does not allow to reach firm conclusions, however the results of our analysis highlight that most of current suggested benefits of ivermectin are based on potentially biased estimates reported by studies with significant methodological limitations. Further research is needed to confirm or reject the effects of ivermectin on patient important outcomes.

There is an urgent need for high quality research both in health emergencies and in health relevant priorities in non-emergency settings. Those involved in evidence production should prioritize quality over quantity and speed to provide trustworthy information that is useful for decision-making. Although countries have capacities to conduct trials, and there exist global standards of quality assurance in clinical trials [68–70], a global coordinating mechanism is needed to streamline and harmonize research findings on an international scale.

5. Conclusions

Ivermectin may not improve clinically important outcomes in patients with COVID-19 and its effects as a prophylactic intervention in exposed individuals are uncertain. Previous reports concluding important benefits associated

FOI 26-3132 - Document 5

with ivermectin are based on potentially biased results reported by studies with substantial methodological limitations. Further research is needed.

Acknowledgments

We would like to thank Victoria Stanford for contributing to the writing of the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jclinepi.2021.12.018.

CRediT authorship contribution statement

Ariel Izcovich: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Sasha Peiris:** Data curation, Writing – original draft, Writing – review & editing. **Martín Ragusa:** Investigation, Data curation, Writing – review & editing, Visualization. **Fernando Tortosa:** Investigation, Data curation, Writing – review & editing, Visualization. **Sylvain Aldighieri:** Writing – review & editing, Project administration, Funding acquisition. **Ludovic Reveiz:** Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Review Article

The multitargeted drug ivermectin: from an antiparasitic agent to a repositioned cancer drug

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Received January 15, 2018; Accepted January 26, 2018; Epub February 1, 2018; Published February 15, 2018

Abstract: Drug repositioning is a highly studied alternative strategy to discover and develop anticancer drugs. This drug development approach identifies new indications for existing compounds. Ivermectin belongs to the group of avermectins (AVM), a series of 16-membered macrocyclic lactone compounds discovered in 1967, and FDA-approved for human use in 1987. It has been used by millions of people around the world exhibiting a wide margin of clinical safety. In this review, we summarize the *in vitro* and *in vivo* evidences demonstrating that ivermectin exerts antitumor effects in different types of cancer. Ivermectin interacts with several targets including the multidrug resistance protein (MDR), the Akt/mTOR and WNT-TCF pathways, the purinergic receptors, PAK-1 protein, certain cancer-related epigenetic deregulators such as SIN3A and SIN3B, RNA helicase, chloride channel receptors and preferentially target cancer stem-cell like population. Importantly, the *in vitro* and *in vivo* antitumor activities of ivermectin are achieved at concentrations that can be clinically reachable based on the human pharmacokinetic studies done in healthy and parasited patients. Thus, existing information on ivermectin could allow its rapid move into clinical trials for cancer patients.

Keywords: Ivermectin, cancer, drug repurposing

Introduction

The antiparasitic drug ivermectin was initially approved in humans in 1987 to orally treat onchocerciasis, also known as river blindness, caused by the blackfly-transmitted parasite *Onchocerca volvulus* in poor populations around the tropics, mostly in West and Central Africa [1, 2]. In humans, its use has improved the nutrition, general health and well-being of billions of people worldwide since it was first used to treat onchocerciasis. Not only that, but in veterinary medicine, ivermectin is used to treat billions of livestock and pets around the world, helping to boost production of food and leather products, as well as to keep billions of companion animals, particularly dogs and horses, healthy. Nowadays, ivermectin by its own has produced sales greater than US$1 billion/annum during the past two decades [3] and is annually taken by close to 250 million people [1].

Ivermectin belongs to the group of avermectins (AVM), which is a group of 16-membered macrocyclic lactone compounds discovered in 1967 in the Japanese Kitasato Institute [1, 3] in fermentation broths of actinomycetes cultures with the fungus *Streptomyces avermitilis* [4-6]. AVM family members include, among others, selamectin, abamectin, monoxidectin and ivermectin (**Figure 1**), all of which differ from the antibacterial and antifungal 16-membered macrocyclic lactones by owning a bisoleandrosyloxy substituent at the C13 [3]. Ivermectin is the most commonly employed compound from the AVM group, being a more potent and safer semi-synthetic mixture of the two AVMs 22,23-dihydroavermectin-B_{1a} and dihydroavermectin-B_{1b}, at a reason of 4:1, respectively [1]. Since 1981 ivermectin has been employed for agriculture, veterinary and aquaculture purposes [1], and is recognized as nematocidal, acaracidal and insecticidal [3, 7]. The antiparasitic efficacy of ivermectin is not limited to onchocerciasis, since it is also effective for filarial infections such as those caused by *Wuchereria bancrofti*, *Brugia malayi*, *Loa loa*, *Mansonella perstans*, and *Mansonella ozzardi* [7]. It also eradicates gastrointestinal parasites, including

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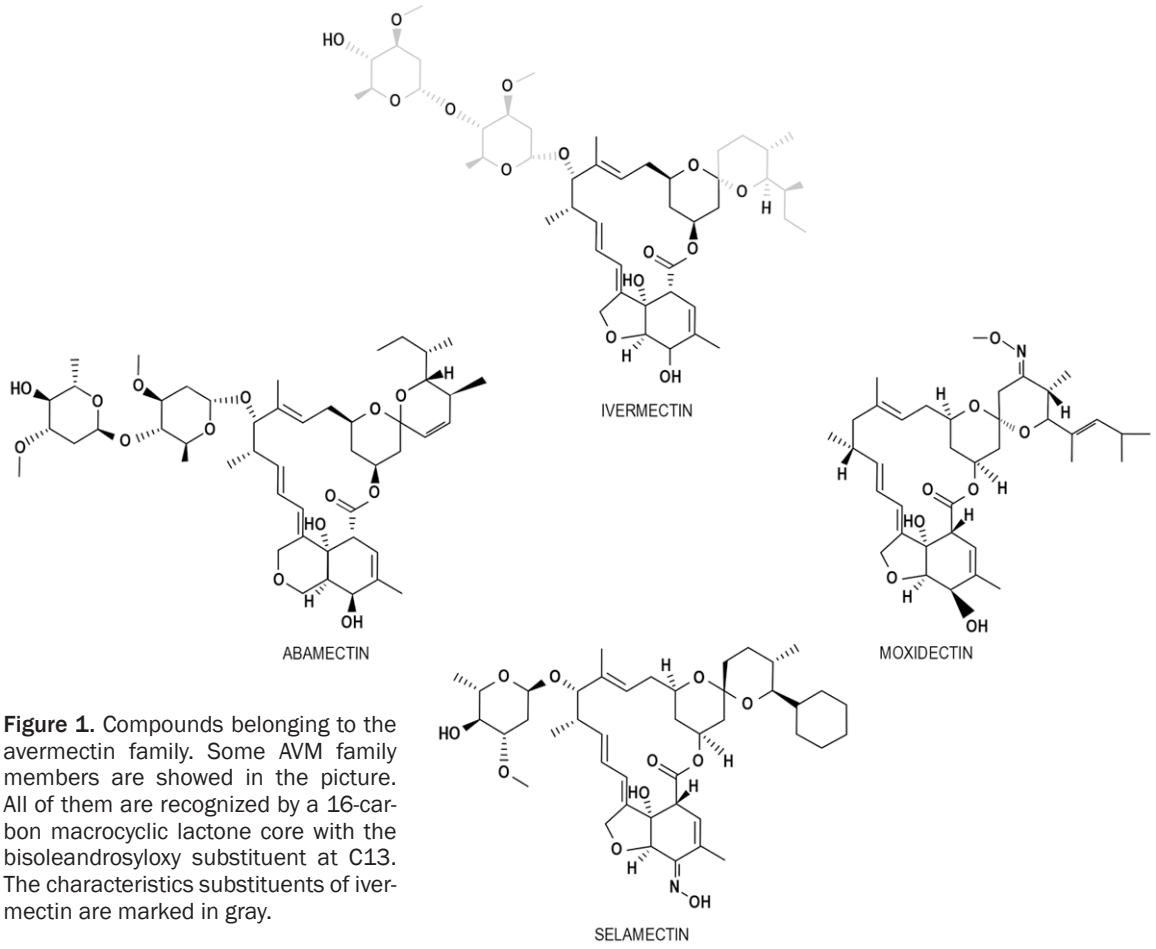


Figure 1. Compounds belonging to the avermectin family. Some AVM family members are showed in the picture. All of them are recognized by a 16-carbon macrocyclic lactone core with the bisoleandrosyloxy substituent at C13. The characteristics substituents of ivermectin are marked in gray.

Ascaris lumbricoides, *Strongyloides stercoralis*, *Enterobius vermicularis*, *Trichuris trichiuria*, and *Ancylostoma duodenale* [7]. Besides, it is employed to treat trypanosomiasis, malaria, leishmaniasis, scabies and head lice [1]. In parasites and helminths, ivermectin, as well as the rest of AVMs, increases the activity of γ-aminobutyric acid (GABA) receptors or glutamate-gated chloride ion channels (Glu-Cl) [3, 4, 6], which blockades the signal between neuron and muscle [3].

In mammals, GABA-sensitive neurons are secured by the blood-brain barrier (BBB) within the central nervous system (CNS), protecting vertebrates against potential harmful effects of AVMs [3, 6]. In support of this statement, subpopulation of collie dogs that have defective function of the multidrug resistance (MDR) protein (commonly a 4 base-pair deletion of the *mdr-1* gene which produces a stop codon), which is an integral part of the BBB and functions as a drug-transport pump in the BBB, have increased neurotoxicity to ivermectin [8]. On the other hand, invertebrates are dose-dependent susceptible due to the widespread allocation of Glu-Cl channels, in whom ivermectin induces the opening of GABA-regulated Cl^- channels that generates an influx of Cl^- [1, 7]. The resulting hyperpolarization impedes the phosphorylation of the regulatory light chain of myosin II by PAK1 [9], promoting paralysis of somatic muscles with concomitant uncoordinated movement, starvation due to inhibition of pharyngeal pumping, and death [1, 3, 5, 7]. Certainly, the affinity of ivermectin for the parasite is 100 times greater than for the mammalian brain, but at least in onchocerciasis, ivermectin action is mostly restricted to the microfilariae stage of *Onchocerca volvulus* [7] as the macrofilariae form does not require pharyngeal pumping to survive [1]. The rate of reduction in microfilarial worms is close to 98% with only two weeks of ivermectin administration, an effect maintained within the next 12 months [1].

FOI 26-3132 - Document 5

Current use and dosage as an antiparasitic drug

In humans, the most used dose of ivermectin for onchocerciasis, strongyloidiasis and enterobiasis ranges between 150 to 200 μg/kg [10-12], while it is used at higher doses of 400 μg/kg for lymphatic filariasis [13]. It is noteworthy the report of a clinical trial on the use of ivermectin for patients with spinal damage and muscle spasms where the drug was administered up to 1.6 mg/kg subcutaneously twice a week for 12 weeks [14].

Toxicity

This compound has a wide margin of safety in ruminants, pigs and equine, as well as in most of the dog breeds [15, 16]. The acute toxicity of ivermectin has been investigated in various species of animals. The signs of toxicity were similar after oral and intraperitoneal administration in rats and mice, and the effects consisted in ataxia, tremors, and reduced activity [17]. In early stages of development, ivermectin at doses of 0.4-0.8 mg/kg in mice, 10 mg/kg in rats, and 3-6 mg/kg in rabbits, increased the incidence of cleft palate, but it was not considered as embryotoxic since the frequency of anomalies was very low [18]. The toxic effects have been related to its interaction with the P-glycoprotein, which limits its access to the CNS. The absence of this protein determines the accumulation of ivermectin in the brain of transgenic mice that do not express it. Finally, in adult Rhesus monkeys that ingested it daily for 16 days at 1.2 mg/kg, no undesirable effects were detected [18].

There are several toxicological reports of ivermectin in different species. The lethal dose 50 (LD50) reported in mice [19] is 25 mg/kg administered orally, whose human equivalent dose (HED) is 2.02 mg/kg. The LD50 increases up to 30 mg/kg when this compound is administered intraperitoneally in mice (HED 2.43 mg/kg). For rats the average lethal dose is 50 mg/kg orally (HED 8.01 mg/kg) and 55 mg/kg intraperitoneally (HED 8.91 mg/kg). In rabbits it is 406 mg/kg in topical application, while in dogs it is 80 mg/kg administered orally (HED 43.24 mg/kg) [20]. Clearly, it seems that the higher the phylogenetic scale the lower toxicity by ivermectin. These data are in accord with the findings in a review paper on avermectins poisoning (14 on suicidal attempt). In this retrospective review, among 18 patients exposed to abamectin and one to ivermectin, 15 were poisoned by oral ingestion. Four were asymptomatic and 8 had minor symptoms with a mean ingestion of 23 mg/kg (range in 4.2-67 mg/kg). Seven patients manifested severe symptoms, such as coma (seven), aspiration with respiratory failure (four), and hypotension (three), after a mean ingestion of 100.7 mg/kg avermectin (15.4 mg/kg for ivermectin and 114.9 mg/kg for abamectin). All 7 seven patients received intensive supportive care; 1 patient died 18 days later as a result of multiple organ failure [21].

In humans it is considered that ivermectin generates low levels of toxicity because its targets are confined within the CNS. Indeed, most patients treated with ivermectin have no side-effects other than those caused by the immune and inflammatory responses against the parasite, such as fever, pruritus, skin rashes and malaise [7, 22], and when present, they appear within 24-48 h after treatment [23]. Certainly, moderate symptoms such as arthralgia, dizziness, fever, skin edema, dyspnea and hypotension may be more related with the microfilarial load in the patient rather than with the intrinsic toxicity of ivermectin [24]. Reports on cases of encephalopathy in patients co-infected with onchocerciasis and lymphatic filariasis after 48 h of treatment with ivermectin can be found in the literature [25], but it is believed that this adverse reaction is due to the obstruction of the microcirculation of the brain by the accumulation of dead or paralyzed parasites, which leads to brain embolism [26].

In conclusion, the immense number of patients who have been treated with ivermectin shows that it is a safe and a well-tolerated drug. Beyond the side effects attributable to the immunological and inflammatory reaction elicited by dying or death parasites, there are sympathetic signs related to ivermectin intoxication, including tremors, mydriasis, sialorrhea, motor incoordination and coma [27].

Pharmacokinetics

The pharmacokinetics of ivermectin have been widely studied in various mammals, including humans; is a fat-soluble compound, with a distribution volumen of 46.9 L; it has a mean peak

Table 1. Pharmacokinetics of ivermectin in healthy and parasited subjects

Condition	Doses (mg/kg)	Route	C_{max} (ng/mL)	T_{max} (h)	AUC (μg/h/mL)
Parasitic infection	0.1-0.2	Oral	52.0	5.2	2.852
Healthy	0.35-0.6	Oral	87.0	4.2	1.444
Healthy	0.7-1.1	Oral	165.2	3.6	2.099
Healthy	1.4-2.0	Oral	247.8	4.2	4.547

plasma level of ~4 h after oral administration with a second peak at 6-12 h because of enterohepatic recycling [1], and possesses an oral clearance of 1.2 L/h [7]. With a plasma protein binding of 93% [7], this drug experiences low biotransformation within the organism [4-6]. The maximum concentration in plasma is reached 4-5 h after its oral administration; its half-life is approximately 19 h and is metabolized in the liver by the cytochrome CYP1A and CYP3A4 complexes, generating 10 metabolites, mostly demethylated and hydroxylated. Its excretion is mainly by feces and only 1% is excreted in the urine [28]. **Table 1** shows the pharmacokinetic data of ivermectin in humans infected with parasites, as well as in healthy humans treated with various doses of ivermectin [28, 29]. According with **Table 1**, the molar concentrations achieved taking into account the total exposure of the drug measured by the area under the curve (systemic exposure) in healthy individuals or patients treated for parasitic diseases are:

3.25 μM/h in parasitized patients with a dose of 0.1-0.2 mg/kg, 1.64 μM in healthy subjects with a dose of 0.35-0.6 mg/kg, 2.4 μM/h in healthy subjects with a dose of 0.7-1.1 mg/kg, and 5.2 μM/h in healthy subjects with a dose of 2 mg/kg.

Drug repurposing in cancer

Drug repurposing, drug redirecting or drug reprofiling is defined as the identification of novel usages for existing drugs. Both development risks and costs, as well as safety-related failure, are reduced with this approach because such drugs have well-known formulation development, *in vitro* and *in vivo* screening, as well as pharmacokinetic and pharmacodynamic profiles. Also, the first clinical phases of many drugs had been completed and can be bypassed to reduce several years of development. Therefore, drug repurposing has the potential to reduce the whole process up to 3-12 years, and consequently, the potential recycle of compounds towards a new indication is an attractive opportunity for patients on need [30]. The relevance of drug repositioning in research is demonstrated with the fact that since the first publication of the subject back to 2004, there are more than 500 papers about it until 2013, at least in PubMed [31]. However, the majority of repositioned agents were discovered before starting with systematic efforts on 2006 to identify drugs with potential additional use, suggesting a serendipitous detection [30, 32, 33]. Currently, other ways to identify compounds with repositioning potential are informed insights and platforms established to identify *in silico* repositioning opportunities [32, 34]. Furthermore, with the recently assembly of the Drug Repurposing Hub, an online repurposing library that systematically classifies a collection of clinically tested compounds from existing databases, now it is possible to easily search and view drugs according to their clinical status, drug indications, or mechanism of action, allowing to rapidly find agents for further evaluation [35].

Important efforts have been made for drug repositioning in cancer. Pantziarka *et al*. have recently summarized on this topic. They report at least 235 non-cancer drugs with proven antitumor activity either *in vitro* or *in vivo*, and among these, 67 (29%) are in the World Health Organization (WHO) list of essential medicines, and 176 (75%) are off-patent [36]. 133 (57%) had human data in cancer patients [36]. Four were listed in clinical guidelines, namely thalidomide, all-trans retinoic acid, zoledronic acid and non-steroidal anti-inflammatory drugs (NSAID) [36]. Of note, at least 3 drugs have shown a survival benefit in randomized trials: cimetidine (colorectal cancer), progesterone (breast cancer) and itraconazole (lung cancer) [36]. Few examples of drug-target networks analyses show that both simvastatin and ketoconazole are anti-proliferative compounds in breast cancer [32], while gene expression profiles suggest that topiramate can be used to treat small-cell lung cancer and that sirolimus can be useful for glucocorticoid-resistant acute

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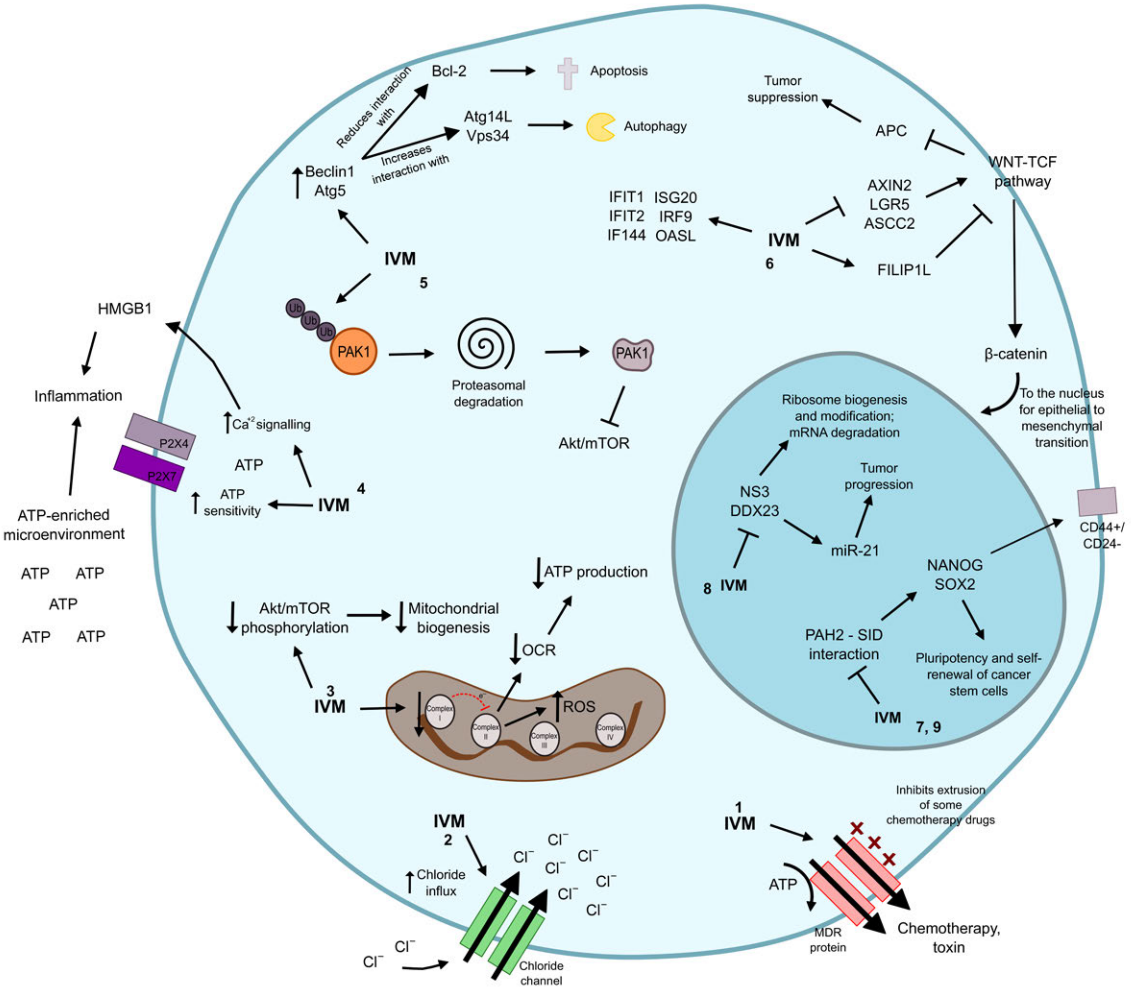


Figure 2. Antitumor mechanisms of ivermectin. 1. Ivermectin inhibits the P-glycoprotein pump, that induces a multidrug phenotype in the cancer cell. 2. Ivermectin acts as an ionophore and up-regulates chloride channels to generate apoptosis and osmotic cell death. 3. By decreasing the function of the mitochondrial complex I, ivermectin limits the electronic movement in the oxidative phosphorylation pathway that stimulates oxygen consumption rate to generate ATP for the cell. Concomitantly there is a reduction in the phosphorylation levels of Akt, impacting in the mitochondrial biogenesis process. Furthermore, alterations in the mitochondrial machinery are related with increased levels of reactive oxygen species that damage DNA. 4. Ivermectin induces ICD through the stimulation of an ATP- and HMGB1-enriched microenvironment, which promotes inflammation. This drug also increases ATP sensitivity and calcium signals in P2X membranal receptors, particularly P2X4 and P2X7, to induce ATP-dependent immune responses. 5. Ivermectin promotes the poly-ubiquitination of the kinase PAK1, which directs it to degradation in the proteasome. Defective PAK1, in turn, inhibits the Akt/mTOR pathway. At the same time, ivermectin stimulates the expression of Beclin1 and Atg5, both related with induction of autophagy and reduces the function of the negative regulator of apoptosis Bcl-2. Together, this generates autophagy and apoptosis. 6. Ivermectin represses AXIN2, LGR5 and ASCL2, all of them positive regulators of WNT-TCF while promotes the repressor of the WNT signaling FILIP1L. Concomitantly, ivermectin promotes the expression of several IFN-related genes, such as *IFIT1*, *IFIT2*, *IF144*, *ISG20*, *IRF9* and *OASL*. 7. Ivermectin modifies the epigenetic signature and the self-renewal activity in the malignant cell due to its ability to mimic the SIN3-interaction that binds to the PAH2 motif of the cancer-associated deregulators SIN3A and SIN3B. SIN3A naturally induces NANOG and SOX2, which are stimulants of stem cell pluripotency. 8. Ivermectin limits the function of the RNA helicases NS3 and DDX23, both of which are related with ribosome biogenesis and post-transcriptional modifications, as well as with mRNA degradation. DDX23 acts as a promoter of miR-21, which is a well-recognized stimulator of tumor progression. 9. Ivermectin inhibitis preferentially the CSC population and up-regulates pluripotency and self-renewal genes NANOG, SOX2 and OCT4. IVM: ivermectin; ATP: adenosine triphosphate; OCR: oxygen consumption rate; ROS: reactive oxygen species.

lymphocytic leukemia [35]. On the other hand, drugs that were first formulated to treat cancer might also be useful to treat non-malignant diseases. That is the case of inhibitors of histone

Table 2. Summary of the antitumor targets of ivermectin

Target	Effect	References
MDR protein	Inhibition	[39]
Chloride channel	Increase of activity	[44]
Akt/mTOR pathway	Inhibition	[47]
P2X7/P2X7 receptors	Activation	[50, 51]
PAK1 protein	Inhibition	[9, 54]
WNT-TCF pathway	Inhibition	[57, 58]
SIN3 domain	Inhibition	[59]
NS3 DDX23 helicase	Inhibition	[64]
Nanog/Sox2/Oct4 genes	Downregulation	[67]

deacetylase enzymes that are approved for T-cell lymphoma, but are prospective targets for malaria, leishmaniasis and trypanosomiasis [37].

Molecular mechanisms of the antitumor effects of ivermectin

Despite the relatively short time on which ivermectin has been investigated as a drug for cancer repositioning, a number of molecular mechanisms of action have been discovered (**Figure 2** and **Table 2**). Among these are the following:

Ivermectin as an inhibitor of the multi-drug resistance (MDR) phenotype

The firsts reports on the potential antitumor activity of ivermectin appeared almost 20 years ago, and these were somehow linked with the recently discovered basis for the MDR phenotype at that time. Gros P *et al*. in 1986 reported the isolation of DNA clones complementary to the cellular messenger RNA transcripts of *MDR* genes, and showed that high-level expression of a full-length complementary DNA clone in an otherwise drug-sensitive cell confers a complete MDR phenotype [38]. Later on, Didier and Loor proved by a short-term assay the P-glycoprotein function inhibition, which measures the restoration of the retention of two P-glycoprotein probes in MDR cells to their parental cells, concluding that ivermectin is also a substrate and an inhibitor of P-glycoprotein [39]. Though not named as ivermectin, avermectin IB1 showed the ability to reduce tumor growth *in vivo* by 50% at day 5 at dose of 1 mg/kg (HED 81 μg/kg) in SHK male mice bearing a solid Ehrlich carcinoma. In addition, at the same dose it inhibits the growth of the carcinoma cell line 755 (C57/BL6 male mice), and the tumor growth inhibition value reached a maximum when avermectin B1 was injected on day 3 after tumor inoculation. Based on the fact that ivermectin inhibits multidrug resistance in tumors, avermectin IB1 was tested with vincristine in the Ehrlich carcinoma, with results indicating that the antitumor effect of vincristine is greatly increased when avermectin 1B1 is administered after vincristine [40]. No further studies have exploited the anti-MDR effect of ivermectin. Nevertheless, the search for novel strategies and/or schedule optimization of MDR inhibitors continues [41], which suggests that there is still room for investigating ivermectin roles in reversing or preventing the development of the MDR phenotype.

Ivermectin as an ionophore drug

The term 'ionophore' was first used in 1967 in reference of the ability of organic molecules to bind metal cations and to form lipid soluble complexes that facilitate their transport across cellular membranes. Thus, ionophores can diffuse back and forth between the extracellular and intracellular spaces, or may remain in the plasma membrane as their transport metal ions between intracellular and extracellular spaces [42]. Ionophore antibiotics act by generating pores in biological membranes that dramatically alter the ionic household of cells. Salinomycin is an example of an ionophore antibiotic which generates ion channel-like structures that exhibit strong selectivity for K^+, but other monovalent cations are also conducted (e.g., Na^+ and H^+) [43]. Traditionally, the cell-killing activity of ionophore antibiotics is thought to originate from profoundly deregulating osmosis, as well as from direct cytotoxic effects of the altered biochemical landscape. It is known that malignant cells tend to upregulate chloride channels, which potentially could mark them as more sensitive to alterations in chloride flux, and that an unbalance in intracellular chloride concentrations affects intracellular Ca^{2+} levels, as well as pH and cell volume, which can lead to apoptosis in the affected cell [44].

In line with these statements, in a screen of a small chemical library of antibiotics and metabolic regulators to identify anti-leukemia compounds, Sharmeen *et al*. found that ivermectin

induces cell death at low micromolar concentrations (IC50 of 10 μM) in HL60, KG1a, and OCI-AML2 acute myeloid leukemia cell lines, as well as in primary patient samples. Cell death was caspase-dependent and interestingly, normal hematopoietic cells were much less sensitive to ivermectin as it did not induce apoptosis at concentrations up to 20 μM. Similar effects were seen when leukemia and normal cells were tested for clonogenicity. They also showed that increased chloride influx correlated with cell death and changes in both cell size and cell hyperpolarization, as these effects were much more marked in sensitive leukemia cells as compared to normal cells. Interestingly, there was a synergistic or additive interaction in OCI-AML2 and U937, but not in normal cells, when they treated with ivermectin plus cytarabine or daunorubicin [44].

Ivermectin as an inductor of mitochondrial dysfunction and oxidative damage

On the basis that anthelmintics or antibiotics may target mitochondria in mammalian cells [45, 46], ivermectin was tested in glioblastoma cell lines to identify whether its antitumor effect occurs via inhibition of mitochondrial biogenesis or function. As expected, ivermectin inhibits in a dose-dependent manner the basal and maximum oxygen consumption rate (OCR), most likely by decreasing the enzyme activity of respiratory complex I but not II, IV or V, and consistent with that, both the membrane potential and electrochemical proton gradient decrease while a significant increase in mitochondrial superoxide and decreased ATP are observed. Furthermore, by establishing a subline of the U87 cell line deficient in mitochondrial respiration it was proved that under these conditions ivermectin was unable to induce cell death, as well as when these cells were co-treated with the antioxidants alfa-tocopherol or mannitol. These effects were tracked down by studying the Akt/mTOR pathway, which at least in part controls mitochondria biogenesis and function. Results showed that ivermectin decreases phosphorylation of Akt (S473), mTOR (S2481) and the ribosomal S6 protein (rS6) in U87, T98G and HBMEC cells, indicating that ivermectin inhibits the Akt/mTOR pathway [47]. The effect of ivermectin upon mitochondria and oxidative damage has been recently corroborated in a study on renal cancer cell lines, where it was demonstrated that renal cancer cells do have higher mitochondrial mass and basal and maximal OCR [48]. As in the Liu *et al*. study, ivermectin in this model also decreased mitochondrial membrane potential as well as basal and maximal respiratory capacities. Of note, ivermectin significantly increased intracellular ROS and 8-OHdG levels, suggesting that the antitumoral effects of ivermectin are related to oxidative stress and DNA damage. This was confirmed by the abolition of the inhibitory effect of ivermectin in these renal cancer cell lines when co-treated with acetyl-L-carnitine (ALCAR) or N-acetyl-L-cysteine (NAC), a stimulant of mitochondrial biogenesis and an antioxidant, respectively [48].

Ivermectin as an inductor of immunogenic cell death (ICD)

Immunogenic cell death (ICD) is characterized by the presence of damage-associated molecular patterns (DAMPs) such as the membranal exposure of calreticulin and the release of both ATP and high-mobility group box 1 (HMGB1) into the extracellular space, which are then recognized by immune cells to elicit antineoplastic activities [49]. Exogenous ATP regulates defense through P1, P2X and P2Y purinergic receptors [50]. However, recently P2X7 overexpression has been correlated with promotion of both tumor growth and metastases [50]. Although within the tumor microenvironment ATPases such CD39 and CD73 degrade ATP to its immunosuppressive form, adenosine, ivermectin can surpass their effect by a potent induction of both HMGB1 and ATP extrusion which, in turn, induces inflammation [50]. Certainly, it has been reported in triple-negative breast cancer cells (TNCB) that ivermectin allosterically potentiates P2X4/P2X7- and caspase-1-mediated ICD due to the stimulation of an ATP-enriched tumor microenvironment, disrupting the balance between the survival and cytotoxic roles of purinergic signaling in malignant cells, which also induces autophagy [50]. One additional work done with human monocyte-derived macrophages corroborated the ivermectin association with PX24 and P2X7 receptors. Such research demonstrated that ivermectin increases ATP sensitivity and delays current deactivation after ATP wash-out in PX24 receptors, which together with the augmentation of ATP-induced currents and Ca^{2+}

signals in P2X7 receptors, suggests that ivermectin may stimulate ATP-dependent immune responses [51]. Altogether, literature indicates that ivermectin could promote and potentiate ICD at the tumor microenvironment.

Ivermectin as an inductor of autophagy

Autophagy, a self-degradative catabolic pathway is characterized by formation of double-membrane autophagosomes, which sequester excess or defective organelles and fuse with lysosomes for degradation of enclosed materials in the lysosome to mobilize energy and nutrients under certain cellular stimuli such as starvation, developmental transitions, hypoxia and/or oxidative stress [52, 53]. Ivermectin in ovarian and glioblastoma cancer cell lines promotes ubiquitination-mediated degradation of the oncogenic kinase PAK1 [1, 47], a key protein in cytoskeletal reorganization and nuclear signaling for tumor growing in more than 70% of all human cancers [1]. PAK1 downregulation in turn blockades the repressor of autophagy Akt/mTOR as evidenced by decreased phosphorylation of Akt, mTOR, p70S6K and 4EBP1 via direct interaction of PAK1 with Akt [54]. In fact, one work with multiple breast cancer cell lines treated with ivermectin showed its autophagy-promoter role by the formation of acidic vesicular organelles, with double-membraned autophagosomes by transmission electronic microscopy, and with the promotion of the expression levels of the autophagy-related proteins Beclin 1 and Atg5 in a dose-dependent fashion. Ivermectin, in turn, increases the interaction of Beclin 1 with positive regulators of autophagy, specifically Atg14L and Vps34, while diminishes its interaction with negative regulators such as Bcl-2 [54]. All together it is demonstrated that the ivermectin autophagic effects in this model results from inhibition of the PAK1/Akt/mTOR pathway.

Ivermectin as an inhibitor of the WNT-TCF pathway

The antiproliferative function of ivermectin has been widely documented. A study aimed to find out drug candidates for repositioning with the ability to block the WNT-TCF signaling, which inactivates the tumor suppressor APC in several sporadic human cancers and stimulates the constitutive activation and translocation into the nucleus of β-catenin during neoplastic transformation [55, 56], revealed that ivermectin at low micromolar concentrations performs anti-WNT-TCF response in cancer cells [57]. The authors showed the efficacy of ivermectin to inhibit BrdU incorporation in colon cancer, glioblastoma and melanoma cell lines, indicating repression in cell proliferation [57]. Besides, they demonstrated upregulation of activated caspase 3 and repression of the positive WNT-TCF targets *AXIN2*, *LGR5*, and *ASCL2* in DLD1 and Ls174T colon cancer cells [57], opening the possibility to use ivermectin to block WNT-TCF-dependent cancers, such as those from breast, skin, lung and intestine [1, 57]. Moreover, after investigating the ivermectin anti-clonogenic activity by analyzing spheroid formation, they showed that pre-treatment of such cell lines with ivermectin diminishes clonal floating spheroids by up to 73%, which together with the repression of the positive cell cycle regulator cyclin D1, suggests a limiting cancer stem cell formation driven by ivermectin [57]. In fact, ivermectin role as an WNT-TCF inhibitor led to use it as a positive control of such pathway in one research that screened a library of plant and microorganism natural compounds, which confirmed ivermectin suppressor activity by transcriptomic analysis that showed an upregulation of the repressor of WNT signaling *FILIP1L* up to 10-fold by the use of this compound [58]. Such study also revealed the increase of the interferon-responsive genes *ISG20*, *IFIT1*, *OASL*, *IRF9*, *IF144*, and *IFIT2*, in colon cancer cells [58]. Interestingly, it has been suggested the employment of interferon to inhibit WNT-TCF pathway [58].

Ivermectin as an epigenetic modulator

Other recognized field of action of ivermectin in cancer involves epigenetic regulation. One work done with the breast cancer cell line MDA-MB-231 reported the functional effect of ivermectin after an *in silico* screen of 2,000 FDA-approved small molecule drugs. The authors evaluated the ability of ivermectin to mimic the SIN3-interaction domain (SID), which naturally binds to the PAH2 motif belonging to the breast cancer-related epigenetic deregulators SIN3A and SIN3B [59]. By nuclear magnetic resonance they demonstrated that ivermectin indeed blocks the PAH2-SID interaction [59]. Furthermore, since SIN3A is part of a complex that positively regulates the stem cell and self-

Table 3. Antitumor effects of ivermectin *in vitro*

Tumor type	Cell lines	[μM]	Effects	Reference
Human leukemia	OCI-AML2	5,	Induces cell death through upregulation of ROS.	[44]
	HL60	10,		
	U937	15,		
	K61a	20		
Prostate cancer	DU145			
	PPC-1			
Human glioblastoma	U87	1,	Induces growth inhibition, apoptosis and anti-angiogenesis.	[47]
	T98G	5,		
		10		
Ovarian cancer	TYK-nu	0.1,	Inhibition of cell proliferation.	[9]
	KOC7C	1,		
	SKOV3	10,		
	RMUG-S	100		
	HEI-193			
Breast cancer	MDA-MB-435,	5,	Stimulates autophagy and inhibition of cell proliferation.	[54]
	MDA-MB-231	10,		
	MDA-MB-468	15,		
	MDA-MB-361	20		
	MCF-7			
	HS578T			
Murine breast cancer	4T1.2	1,	Induces apoptosis and necrosis. Induces autophagy.	[50]
	DDHer2	4,		
		8,		
Murine melanoma	C57BL/6	16	Inhibition of cell proliferation and clonogenic capacity.	
Murine colon adenocarcinoma	CT.26		Increases the amount of ROS.	
Human breast cancer	MDA-MB-231			
	MCF7			
	SKBR3			
Human melanoma	A2058			
	A375			
Human pancreatic cancer	PANC1			
	MiaPaca2			
Human prostate cancer	DU145			
Human head and neck cancer	A253			
Human leukemia	MV411			
Human colon cancer	CC14	0.1,	Inhibits cell proliferation and induces apoptosis.	[57]
	CC36	1,		
	Ls174T	5,		
	HT29	10		
Human glioblastoma	DLD1			
	U251			
Human melanoma	SKMe12			
Murine breast cancer	4T1	0.01,	Inhibition of invasiveness and restoration of tamoxifen sensitivity.	[59]
	MMTV-Myc	0.1,		

Human breast cancer	MDA-MB-231 D3H2LN	1	Inhibition of cell growth and metastases.	
Human breast cancer	MDA-MB-231	0.2, 0.4, 0.8, 1, 5, 8	Preferentially inhibits cell viability and clonogenicity of the stem cell population.	[67]

renewal markers *NANOG* and *SOX2*, the authors analyzed the role of ivermectin to inhibit such genes and proved that in D3H2LN cells at doses of 0.5 μM it reduces *NANOG* and *SOX2* gene expression by 80%, with a decrease between 90-100% in clonogenic tumorsphere growth [59].

Ivermectin as a helicase inhibitor

RNA helicases represent a large family of proteins implicated in many biological processes, including ribosome biogenesis, splicing, translation and mRNA degradation [60]. Members of the DEAD-box family of RNA helicases play important roles in various aspects of RNA processing, including transcription, spliceosome biogenesis, ribosome biogenesis, splicing, nucleocytoplasmic transport, translation and decay [61, 62]. These family members share a conserved core that includes the amino acid sequence D-E-A-D (aspartate-glutamate-alanine-aspartate). They use energy received from ATP hydrolysis to unwind double-stranded RNA, generally act as components of multi-protein complexes, and have diverse functions that depend on their interacting partners. Several DEAD-box RNA helicases are aberrantly expressed in various types of cancer, where they may play important roles in cancer development and/or progression [61, 62]. In 2012, Mastrangelo *et al*. uncovered that ivermectin was an effective inhibitor of the NS3 helicase activity from the Kunjin virus (an Australian variant of the yellow fever virus) by an *in silico* test, and confirmed it by *in vitro* helicase enzymatic assays [63]. On the other hand, a recent study in glioma cell lines has found that the RNA helicase DDX23 regulates the oncogenic miR-21 biogenesis at post-transcriptional level [64], and it is over-expressed in glioma tissues as compared to normal brain, which is associated with poor survival of glioma patients. After the authors assayed a number of drugs known to inhibit viral helicases, they found that ivermectin inhibits the DDX23-mediated potentiation of pri-miR-21 processing that, in turn, decreases the levels of both precursor and mature miR-21, a well-recognized poor prognostic upregulated marker in cancer [65]. The treatment with ivermectin in glioma cell lines both *in vitro* and *in vivo* was able to induce antitumoral effects, which suggests that the antihelicase role of ivermectin can be considered as another mechanism of its anticancer effect.

Ivermectin as a stem-cell cancer inhibitor

In 2009, Gupta *et al*. performed a high-throughput screening to discover selective inhibitors of cancer stem cells (CSCs), and found that salinomycin reduces the proportion of CSCs by >100-fold relative to paclitaxel. Besides, they showed that salinomycin inhibits mammary tumor growth *in vivo*, and that it induces increased epithelial differentiation of tumor cells accompanied by the loss of expression of breast CSC genes [66]. As salinomycin is an antiparasitic drug for veterinary use only, our group searched similar compounds for human use that could also act as selective inhibitors of CSCs. Our results showed that ivermectin has high similarity with salinomycin (similarity of 0.78), and therefore we hypothesized that the antiparasitic drug ivermectin could also have similar biological properties as salinomycin [67]. The results of this study showed that ivermectin has growth inhibitory effects upon MDA-MB-231 cells in the range of 0.2-8 μM, and as predicted, ivermectin preferentially inhibits the viability of CSCs-enriched populations ($CD44^+/CD24^-$) and cells growing in spheroids, as compared to bulk cell population. The opposite pattern was observed with paclitaxel where the non-CSCs ($CD44^+/CD24^+$) were sensitive to paclitaxel at nanomolar concentrations, while the inhibition of the stem cell subpopulation was only observed at higher drug

Table 4. Antitumor effects of ivermectin in vivo

Tumor type	Tumor cell line	Days of treat.	Dose mg/kg	Mice	Effects of Ivermectin	Ref.
Murine leukemia	MDAY-D2	10	3, 5, 6 i.p.	NOD/SCID mice	Reduces tumor volume up to 70% in all models	[44]
Human leukemia	K562 OCI-AML2		3 oral			
Human glioblastoma	U87 T98G	21	40 i.p.	SCID mice	Reduces tumor volume up to 50%	[47]
Breast cancer	MDA-MB-231-GFP	10	2.4 i.p.	NOD/SCID mice	Reduces tumor volume up to 60%	[54]
Human glioma	U87MG	42	3, 10 i.t.	Balb/c nude mice	Reduces tumor volume up to 50% at 3 mg/kg. At 10 mg/kg tumors were not detectable	[64]
Human colon cancer	LDL1	21	10	NMRI nude mice	Reduces tumor volume up to 85% (LDL1 cell line). No effect is observed in the tumor TCF-independent cell line (CC14)	[57]
	CC14 HT29		i.p.			

i.t.: intratumoral. i.p.: intraperitoneal.

concentrations. According with this, ivermectin reduces the expression of maintenance of the pluripotency and self-renewal markers Nanog, Oct4 and Sox2 at both mRNA and protein levels [67]. A summary of the molecular mechanisms of the antitumor effects of ivermectin are shown in **Table 2** and **Figure 2**.

Antitumor effects of ivermectin, in vitro and in vivo

There are a number of *in vitro* and *in vivo* preclinical studies where ivermectin demonstrates its efficacy against a wide range of malignant conditions, including solid and hematological malignancies. In breast cancer, ivermectin has been studied in MDA-MB-435, MDA-MB-231, MDA-MB-468, MDA-MB-361, MCF-7, HS578T and SKBR3 cell lines where it demonstrates its ability to inhibit cell proliferation, induction of apoptosis, autophagy and reversion of tamoxifen resistance among other effects [51, 55, 60, 67]. These effects are also reported in cancer cell lines from ovarian, prostate, head and neck, colon, and pancreas, as well as in melanoma [9, 45, 51]. Similar results are also observed in a number of murine cancer cell lines including breast, melanoma and colon [51, 60]. Two studies in glioblastoma cell lines show that ivermectin not only induces growth arrest and apoptosis, but induces anti-angiogenic effects [48, 58], whereas two more studies extend these observation upon myeloblastic acute leukemia cell lines [41, 51]. Of interest, the median concentration used for the *in vitro* treatment from all studies referred in **Table 3** was 5 μM (0.01-100 μM) which is clinically achievable according with the pharmacokinetic data in humans shown in **Table 1**. Regarding the *in vivo* evaluation of ivermectin (**Table 4**), this has been done in immune deficient mice using human acute myeloblastic leukemia, glioblastoma, breast and colon carcinoma, as well as in the murine lymphosarcoma cell line MDAY-D2. These studies show more than a 50% reduction in tumor volumes after ivermectin treatment, which varied from 10 to 42 days of treatment by either oral, intraperitoneal or intratumoral routes (more commonly intraperitoneal). The median dose employed was 5 mg/kg (2.4-40 mg/kg), which is equivalent to 0.40 mg/kg in humans, a dose below to the highest dose safely used in human subjects evaluated so far (2 mg/kg) (**Table 1**). Thus, the

in vitro and *in vivo* results with ivermectin strongly suggest that its antitumor effects in cancer patients can be achieved at feasible doses.

Conclusions

The recognition that drug repositioning is a clever opportunity to accelerate the development of cancer drugs is increasing. So far, at least 235 clinically-approved, non-cancer drugs have proven antitumor activity either *in vitro, in vivo*, or even clinically. Among these, ivermectin, an antiparasitic compound of wide use in veterinary and human medicine, is clearly a strong candidate for repositioning, based on the fact that i) it is very safe, causing almost no side-effects other than those caused by the immune and inflammatory responses against the parasite in infected patients, and ii) it has proven antitumor activity in preclinical studies. On the other hand, it is now evident that the use of very selective "unitargeted" drugs is commonly associated to early development of resistance by cancer cells, hence the use of "dirty" or "multitargeted" drugs is important to explore. In this sense, ivermectin has this potential as it modulates several targets such as the multidrug resistance protein (MDR), the Akt/mTOR and WNT-TCF pathways, the purinergic receptors, the PAK-1 protein, certain cancer-related epigenetic deregulators such as SIN3A and SIN3B, RNA helicase activity, while stimulates chloride channel receptors leading to cell hyperpolarization, and down-regulates stemness genes to preferentially target cancer stem-cell like population, at least in breast cancer. Importantly, the *in vitro* and *in vivo* antitumor activities of ivermectin are achieved at concentrations that can be clinically reachable based on the human pharmacokinetic studies done in healthy and parasited patients. Thus, existing information on ivermectin could allow its rapid move into clinical trials for cancer patients.

Acknowledgements

Mandy Juarez is a student belonging to the Programa de Ciencias Bioquímicas, UNAM. Alejandro Schcolnik-Cabrera is a student belonging to the Plan de Estudios Combinados en Medicina (PECEM), UNAM.

Disclosure of conflict of interest

None.

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Transactions of the Royal Society of Tropical Medicine and Hygiene (2004) **98**, 496–504

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Adverse systemic reactions to treatment of onchocerciasis with ivermectin at normal and high doses given annually or three-monthly

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Received 10 June 2003; received in revised form 28 October 2003; accepted 29 October 2003

KEYWORDS
Onchocerciasis;
Onchocerca volvulus;
Ivermectin;
Cameroon;
Clinical trial;
Tolerance

Summary In Cameroon, a 3-year randomized, double-blind controlled trial was conducted to determine if ivermectin, given at 3-monthly intervals and/or at high doses (800 μg/kg), had a greater effect on adult *Onchocerca volvulus* than standard annual doses of 150 μg/kg. Adverse reactions were recorded and analysed in a logistic regression model with random effects to assess the influence of the dose and rhythm of treatment on their occurrence. After the first dose, 3-monthly treatment was associated with a clearly reduced risk of reactions, especially oedematous swellings, pruritus and back-pain. Oedematous swellings and subjective ocular troubles were found to be associated with high doses of ivermectin. These results reinforce former parasitological conclusions that it would be desirable to evaluate the feasibility and effects on transmission of large-scale 3-monthly treatments with standard doses of ivermectin for onchocerciasis control. Owing to the unexpected ocular reactions, the use of high doses to counteract any future resistance of *O. volvulus* to ivermectin should be considered with caution.
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1. Introduction

In 1993, the WHO estimated that 18 million people suffered from onchocerciasis, over 95% of them in sub-Saharan Africa and the rest in six Latin American countries and the Yemen (WHO, 1995). Control of onchocerciasis in Africa is currently based on mass administration of ivermectin (Mectizan®, Merck & Co., Inc., Whitehouse Station, NJ, USA) at an annual dose of 150 μg/kg to all eligible persons in communities where the disease is hyper or

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doi:10.1016/j.trstmh.2003.10.018

mesoendemic. In Latin America, the same dosage is given on a 6-monthly basis.

At 150 μg/kg annually, ivermectin does not kill the adult *Onchocerca volvulus* which, after 3–6 months of embryostasis, resume production of microfilariae (mf) (Duke et al., 1991). When repeated the treatments seem to cause an irreversible decline in mf production of 30% per treatment (Plaisier et al., 1995), but this would not prevent the intensity of transmission from increasing again some time after the distribution, thus preventing control of the parasite (Winnen et al., 2002).

We undertook a double-blind, randomized controlled trial in Cameroon, to evaluate if higher and/or more frequent doses of ivermectin would have a greater effect on the adult worms and mf of *O. volvulus* than standard doses. Of four groups of patients, the reference group received the standard dose of 150 μg/kg annually (Group 150 × 1); the other three received respectively: 150 μg/kg 3-monthly (Group 150 × 4); high doses (400 μg/kg then 800 μg/kg) annually (Group 800 × 1); and the same high doses 3-monthly (Group 800 × 4). We aimed to investigate if more frequent or higher doses would have greater impact on the adult worms and mf thus leading to a shortening of treatment campaigns.

The design and parasitological results of this trial have been published elsewhere (Gardon et al., 2002) with the conclusion that 3-monthly treatments, whether at 150 μg/kg or at high doses, increased the mortality of adult *O. volvulus* by some 30% and diminished considerably the fertility of the females; and that 3-monthly treatments reduced the concentrations of mf in the skin much more than did annual treatments. The present paper describes the adverse systemic reactions that were encountered during this trial.

2. Methods

2.1. Study area and design

The study took place in the Republic of Cameroon, in 20 villages in the Mbam River valley (a tributary of the Sanaga River), where onchocerciasis was hyperendemic (Macé et al., 1997). Initially it involved 657 men aged 18–60 years, each presenting at least two palpable, presumably *O. volvulus* nodules, but otherwise in good health.

In May 1994, a pre-treatment nodule was excised from each man followed by a suppressive dose of 150 μg/kg ivermectin to avoid the possibility of severe reactions developing after the first dose in those on the high-dose schedules. Each patient was then randomly allotted to one of the four treatment groups. The trial proper began in August 1994 and ended in August 1997, when a second nodule was removed from each patient to assess the effect of the each regimen on the adult worms. As no treatment was given in August 1997, the 3 year follow-up corresponded to three doses of ivermectin (and nine of placebo) for the groups treated annually, and 12 doses of ivermectin for the groups treated 3-monthly.

When the trial started, the highest dose permitted by Merck & Co., Inc. was 400 μg/kg. Six months later, when the results of Awadzi et al. (1995, 1999) became available, we were allowed to increase the high-dose regimens from 400 to 800 μg/kg. Thus our high-dose patients treated annually received one dose of 400 μg/kg and two doses of 800 μg/kg, and those on the 3-monthly regimen received two doses at 400 μg/kg and ten doses of 800 μg/kg. Including the clearing dose (150 μg/kg) given 3 months before the trial proper began, the patients treated annually at 150 μg/kg received a total of 600 μg/kg; and those on high dosage a total of 2150 μg/kg. In the groups treated 3-monthly, those on 150 μg/kg received a total of 1950 μg/kg, and those on high dosage 8950 μg/kg.

2.2. Treatment given and adverse reactions recorded

The trial was conducted double-blind, thanks to Merck & Co. which provided indistinguishable gelatin capsules, containing either ivermectin or placebo. At the outset, each patient was given a plastic name card, identifying him to the medical team for ivermectin treatment or consultation about reactions. Ivermectin was given in the morning under the strict control of one of the investigators. Each patient received his correct dose of ivermectin or placebo given in five gelatin capsules, pre-prepared by the pharmacist in a packet with the patient's name and photograph attached. Neither the patients nor those giving out the drug knew what dose any patient was receiving.

In the afternoon of each treatment day (D0) and on each of the following three days (D1–D3), a medical team moved from village to village, at appointed times, to be at the disposal of patients who presented adverse reactions. The patients were asked to come to well-defined places for free palliative treatment as necessary. Each was instructed to seek another consultation if his reaction to treatment had not settled within 24–48 hours. A member of each treated patient's family was

instructed to inform the team in the event of a domiciliary visit being necessary.

During the first six treatment rounds, full details of all reactions were 'narratively' recorded; but, from the treatment round in November 1995, experience allowed us to keep records on the basis of a list of 12 reactive symptoms and signs or groups thereof (see Tables 1–3). Fever was diagnosed by touch. For analysis, all the complaints recorded before November 1995 were recoded so as to be included in one or other of these 12 symptoms.

2.3. Statistical analysis

When a patient complained of more than one type of reaction at any given round, each reaction was included for analysis. This was so either when a patient showed ≥2 reactions on a given day, or when he came more than once (i.e. on different days), each time with a different sign or symptom. When the patient came more than once for the same sign, only one complaint was considered for analysis.

The first step in the analysis involved comparing the mean number of consultations as a whole, and the mean number of consultations for a given symptom, using the non-parametric test of Kruskal–Wallis. Then, a logistic regression analysis was performed, in which the risk of occurrence of each adverse reaction in the 'non-standard' treatment groups was compared with the risk in the reference group (150 × 1). In this analysis, we took into account the fact that the adverse reactions presented by any one patient at any one treatment round, or over the whole course of the trial, could not be considered as independent events. To take account of the excess variation introduced by this 'individual effect', we used a logistic regression model with random effects. Odds ratios (OR), with their 95% CIs, were calculated for each group and each reaction.

The next step in the analysis was to evaluate the way in which the risk of occurrence of a given symptom at a given round was dependent on the rhythm of treatment, and on the dose. In this analysis, the reference categories were the annual ivermectin treatment and the doses of placebo. As in the previous analysis, we used a logistic regression model with random effects. This analysis allowed us to evaluate, simultaneously and within a single model, the relationship between the dosage level and the interval between dosings, and to adjust the one in relation to the other.

Finally, to focus the analysis on the effect of high doses of ivermectin, we compared the impact of the dose given, after having discarded the treatment rounds in which the patients received placebo; thus no longer using the placebo as the reference, but instead using the standard 150 μg/kg dose.

3. Results

3.1. General information

Of the 657 patients allocated initially to one of the four treatment groups, only 643 actually participated in the first treatment in August 1994. Comparisons of the means of their ages, heights, weights, numbers of palpable nodules and *O. volvulus* microfilarial densities showed that the four groups did not differ significantly (Gardon et al., 2002). After 3 years, 572 patients completed the protocol and received the twelfth and last treatment in May 1997. Three months later, 541 of them submitted to further skin snips and the excision of a second palpable nodule. Over the whole trial, 7237 treatments were given, i.e. 2808 doses of placebo; 2226 of ivermectin at 150 μg/kg; and 2203 of high-level ivermectin (475 at 400 μg/kg and 1728 at 800 μg/kg).

3.2. Causes of exclusion from the cohorts and serious reactions

By August 1997, 24/643 patients, who took their first treatment in August 1994, had died in the interval (seven in Group 150 × 1; eight in Group 800 × 1; two in Group 150 × 4; and seven in Group 800 × 4). None of these deaths was related to treatment (see details in Gardon et al., 2002). Between August 1994 and August 1997, 78 other patients were excluded from the trial: 17 on medical grounds and 61 because they had moved away or missed one treatment round. The proportions of patients lost (overall or for each of the above three specific reasons) did not differ significantly between groups.

No patient developed a serious adverse reaction, none required hospitalization, and, no-one withdrew from the trial because of an adverse reaction.

3.3. Risk of mild adverse reactions in the trial as a whole

Mild adverse reactions began very soon after taking the gelatin capsules. Of 1129 consultations for adverse reactions, 367 (32.5%) took place in the afternoon following treatment the same morning (D0); and 449 (39.8%), 195 (17.3%) and 118 (10.5%) took place on the following three days (D1–D3). This distribution over time was similar in all four treatment groups ($P = 0.69$).

Table 1 Number of consultations for, and risk of occurrence of, various adverse reactions in each of the four treatment groups

Dosage group	150 × 1	800 × 1	150 × 4	800 × 4
Total no. of doses	1878	1965	1783	1750
Occurrence of at least one symptom	283	292 0.96 (0.64–1.44)	255 0.91 (0.59–1.40)	299 1.17 (0.74–1.85)
Total no. of symptoms	456	513	402	452
Sundry aches and pains	23	25 1.07 (0.52–2.18)	13 0.61 (0.27–1.37)	18 0.85 (0.4–1.83)
Joint pains	34	18 0.44* (0.21–0.94)	29 0.85 (0.42–1.70)	32 1.03 (0.52–2.04)
Back and waist pain	42	44 0.79 (0.39–1.60)	22 0.49 (0.23–1.06)	26 0.58 (0.27–1.23)
Headache	89	93 0.88 (0.48–1.62)	79 0.87 (0.48–1.57)	93 0.98 (0.54–1.80)
Sensation of fever	31	46 1.39 (0.72–2.69)	31 0.96 (0.48–1.91)	28 0.89 (0.44–1.79)
Pruritus	95	125 1.24 (0.79–1.95)	103 1.13 (0.70–1.81)	100 1.09 (0.68–1.73)
Oedematous swellings	31	50 1.55 (0.86–2.81)	15 0.49 (0.24–1.02)	25 0.87 (0.45–1.68)
Cutaneous eruptions	15	17 1.07 (0.44–2.60)	23 1.61 (0.69–3.76)	25 1.79 (0.77–4.14)
Subjective ocular troubles	18	31 1.62 (0.72–3.64)	18 1.04 (0.45–2.42)	50 3.30** (1.58–6.96)
Asthenia	33	24 0.71 (0.36–1.42)	34 1.16 (0.59–2.76)	26 0.84 (0.42–1.69)
Abdominal troubles	15	15 0.95 (0.41–2.18)	6 0.42 (0.15–1.20)	4 0.29* (0.09–0.95)
Giddiness	18	8 0.44 (0.16–1.17)	15 0.91 (0.39–2.17)	17 1.03 (0.43–2.46)
Others	12	17 1.34 (0.60–3.02)	14 1.24 (0.53–2.87)	8 0.71 (0.27–1.84)

The odds ratios (95% CI) were calculated using treatment with 150 μg/kg as the reference category.
*$P < 0.05$.
**$P = 0.0017$.

During the 3 years of treatment, the mean number of consultations per patient was the same in each of the four treatment groups: i.e. 1.8 (SE = 2.4) for 150 × 1; 1.8 (SE = 2.9) for 800 × 1; 1.7 (SE = 2.3) for 150 × 4; and 1.9 (SE = 3.0) for 800 × 4 ($P = 0.92$). Over the whole trial, the risk in the non-standard treatment groups of presenting at least one adverse symptom or sign after taking the treatment, was comparable to that observed in the reference group (Table 1), with ORs of 0.96, 0.91, and 1.17. The same applied to each of the individual symptoms, almost all of which appeared with similar frequencies in all four groups. Only arthralgia seemed slightly less frequent in the group 800 × 1 (OR = 0.44, 95% CI 0.21–0.94); whereas abdominal pains were less frequent (OR = 0.29, 95% CI 0.09–0.95), and subjective ocular troubles (transitory blurring of vision, itching or pain of the eye, and in several cases dyschromatopsia) more frequent (OR = 3.30, 95% CI 1.58–6.96) in the group 800 × 4 than in the reference group.

3.4. Changes in the risk of mild adverse reactions over time

Although the results presented above demonstrate only small differences between the four groups, taking the 3 year trial as a whole, things were very different when each treatment round is considered separately. The incidence of adverse reactions diminished steadily over the course of the trial. Overall, they affected 28.6% of patients after the first round of treatment, 20.0% during the course of the first year of treatment, 12.5% in the course of the second year and 8.0% during the third year.

Table 2 Symptoms recorded and the risk of their happening as a function of the ivermectin dosage regimen

	No. of events	Dosage interval		Dose			
		3-monthly[a]		150 μg/kg[b]		800 μg/kg[b]	
		OR (*P*)	95% CI	OR (*P*)	95% CI	OR (*P*)	95% CI
Any one symptom	1129	0.36 (<0.001)	0.25–0.52	4.27 (<0.001)	3.21–5.67	6.30 (<0.001)	4.69–8.46
Sundry pains	79	0.46 (0.021)	0.24–0.89	1.60 (0.19)	0.79–3.23	2.22 (0.018)	1.14–4.30
Joint pains	113	0.88 (0.70)	0.47–1.66	1.57 (0.18)	0.81–3.02	2.09 (0.028)	1.08–4.02
Back and waist pain	134	0.33 (<0.001)	0.18–0.61	2.36 (0.002)	1.36–4.10	2.53 (<0.001)	1.48–4.33
Headache	354	0.64 (0.066)	0.40–1.03	1.95 (0.001)	1.30–2.91	1.84 (0.003)	1.24–2.74
Fever	136	0.38 (<0.001)	0.22–0.66	2.85 (<0.001)	1.62–4.98	3.31 (<0.001)	1.95–5.61
Pruritus	423	0.34 (<0.001)	0.24–0.49	6.04 (<0.001)	4.20–8.68	6.55 (<0.001)	4.63–9.28
Oedematous swellings	121	0.11 (<0.001)	0.067–0.20	77.08 (<0.001)	23.0–257.9	131.4 (<0.001)	39.6–436.0
Cutaneous eruptions	80	0.58 (0.10)	0.31–1.12	6.42 (<0.001)	2.78–14.84	3.19 (<0.001)	3.19–16.55
Subjective ocular troubles	117	0.61 (0.11)	0.34–1.11	2.67 (0.008)	1.29–5.56	8.57 (<0.001)	4.46–16.47
Asthenia	117	0.69 (0.23)	0.38–1.26	2.54 (0.003)	1.39–4.66	1.85 (0.059)	0.98–3.52
Abdominal troubles	40	0.42 (0.093)	0.15–1.16	0.78 (0.63)	0.28–2.17	0.92 (0.86)	0.35–2.43
Giddiness	58	1.15 (0.75)	0.47–2.81	1.46 (0.41)	0.59–3.63	1.07 (0.89)	0.40–2.85
Others[c]	51	0.47 (0.042)	0.22–0.97	2.77 (0.015)	1.22–6.26	2.00 (0.10)	0.87–4.63

The odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated using regression models with random effects.
[a] Reference category: annual interval.
[b] Reference category: dose of placebo.
[c] Insomnia, feeling cold, cramps, nausea, anorexia, paresthesiae, pains in the chest, etc.

This fall-off was observed in all the groups, but in those that received ivermectin annually, it was associated with a recrudescence only when the patients received ivermectin, i.e. at rounds 5 and 9. In both groups treated annually, the frequency of adverse reactions was higher after round 5 than after round 9. After round 5, the proportions of consultations in the groups 150 × 1 and 800 × 1 were 24.5 and 30.9%, respectively; in the groups 150 × 4 and 800 × 4, they were 17.8 and 22.6% (comparison between the four groups: $P = 0.060$). Likewise, after round 9, the proportions of consultations in the groups 150 × 1 and 800 × 1 were 18.0 and 21.5% respectively; in the groups 150 × 4 and 800 × 4, they were only 9.0 and 12.5% (comparison between the four groups: $P = 0.014$).

When the various symptoms are considered separately, this variability over time was particularly noticeable with regard to itching, which was also the most frequent secondary symptom encountered. Taking the study as a whole, the risk of itching developing after a round of treatment seemed to be almost the same in all groups including the reference group, with ORs of 1.24, 1.13, and 1.09 in the groups 800 × 1, 150 × 4 and 800 × 4, whereas in reality the frequency of itching varied greatly as a function of the dosage rhythm (Figure 1). In fact, in the groups treated annually there was a strong recrudescence of itching after rounds 5 and 9, which was the price to be paid for the much lower number of consultations requested after those rounds at which the placebo was administered.

3.5. Study of the respective effects of dosage and of the rhythm of ivermectin treatment on the occurrence of reactions

Analysis shows that taking ivermectin every 3 months was associated with a clearly reduced risk of adverse reactions (OR = 0.36, 95% CI 0.25–0.52), regardless of the dose (Table 2). When considering the various symptoms separately, the analysis shows that the reduction of risk associated with 3-monthly treatments was especially noticeable with regard to oedema (OR = 0.11, 95% CI 0.07–0.20), pruritus (OR = 0.34, 95% CI 0.24–0.49) and back- or waist-pain (OR = 0.33, 95% CI 0.18–0.61). In addition, the risk of joint pains, headache, rash, subjective ocular troubles, asthenia, intestinal troubles and giddiness seemed to be independent of the rhythm of ivermectin treatment.

When compared to treatment with placebo, ivermectin dosage was associated with a marked increase in adverse reactions, whether it was given at the standard dose of 150 μg/kg (OR = 4.3, 95% CI 3.2–5.7) or at high doses (OR = 6.3, 95% CI 4.7–8.5). The manifestations with the highest occurrence after ivermectin treatment were itching, rash, oedematous swellings and fever; and, amongst them, swellings appeared to be more frequently associated with high-dose treatment (OR = 131.4, 95% CI 39.6–436.0) than with standard doses (OR = 77.1, 95% CI 23.0–257.9). Besides these signs, which are classically reported in many studies, the analysis showed that ivermectin treatment was also associated with a risk of occurrence of subjective ocular problems (blurring of vision, changes in colour vision, etc.). In contrast, giddiness and abdominal problems seemed to be independent of the treatment regimen. Throughout the three years of the trial, 2808 doses of placebo were given and the adverse reactions most frequently observed after these treatments were headache (3.7%), pruritus (3.0%), back and waist pain (1.6%), sensation of fever (1.4%), asthenia (1.1%) and joint pain (1.1%).

If one eliminates the placebo dosings from the analysis, one can compare the impact of high

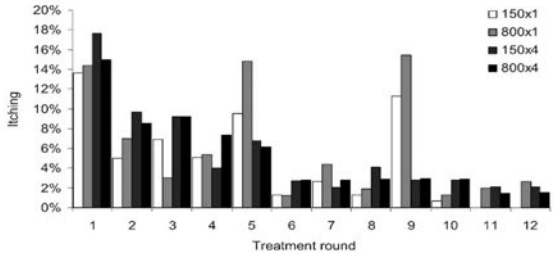


Figure 1 Development over time of the frequency of itching after treatment in the four treatment groups.

Table 3 Symptoms recorded and the risk of their happening as a function of the treatment regimens

	3-monthly treatment[a]		Dose 800 μg/kg[b]	
	OR (*P*)	95% CI	OR (*P*)	95% CI
Any one symptom	0.35 (<0.001)	0.25–0.50	1.24 (0.22)	0.88–1.75
Sundry pains	0.44 (0.012)	0.24–0.83	1.37 (0.32)	0.74–2.55
Joint pains	0.87 (0.65)	0.47–1.61	1.19 (0.56)	0.67–2.10
Back and waist pain	0.32 (<0.001)	0.19–0.56	1.12 (0.68)	0.65–1.94
Headache	0.63 (0.040)	0.41–0.98	1.03 (0.89)	0.67–1.58
Fever	0.38 (<0.001)	0.22–0.65	1.18 (0.53)	0.70–2.02
Pruritus	0.33 (<0.001)	0.24–0.47	1.14 (0.47)	0.81–1.60
Oedematous swellings	0.012 (<0.001)	0.067–0.19	1.76 (0.039)	1.03–2.96
Cutaneous eruptions	0.60 (0.15)	0.30–1.21	1.14 (0.70)	0.58–2.23
Subjective ocular troubles	0.58 (0.070)	0.32–1.05	3.22 (<0.001)	1.76–5.87
Asthenia	0.69 (0.18)	0.40–1.19	0.72 (0.22)	0.43–1.21
Abdominal troubles	0.40 (0.066)	0.15–1.06	1.11 (0.83)	0.43–2.89
Giddiness	1.13 (0.78)	0.47–2.73	0.72 (0.41)	0.33–1.57
Others[c]	0.47 (0.039)	0.23–0.96	0.74 (0.41)	0.37–1.51

The odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated using regression models with random effects.
[a] Reference category: annual interval.
[b] Reference category: dose of ivermectin at 150 μg/kg.
[c] Insomnia, feeling cold, cramps, nausea, anorexia, paresthesiae, pains in the chest, etc.

versus normal dosings (Table 3). In these circumstances, 3-monthly dosage was still associated with a marked decrease in the risk of developing adverse reactions, when compared with annual treatments (OR = 0.35, 95% CI 0.25–0.50). Of the different adverse manifestations, only oedematous swellings (OR = 1.8, 95% CI 1.0–3.0) and subjective ocular problems (OR = 3.2, 95% CI 1.8–5.9) were more frequent after high doses than after the standard dose.

4. Discussion

The safety of ivermectin treatment has allowed it to be used by the African Programme for Onchocerciasis Control (APOC) in mass community-directed treatment with ivermectin (CDTI). The only areas where mass ivermectin treatments against onchocerciasis are associated with problems concerning the management of adverse reactions are those where onchocerciasis is co-endemic with loiasis. In communities where the prevalence of *Loa loa* microfilaraemia is >20%, and the proportion of persons at high risk of severe *Loa*-encephalopathic reactions is sufficiently high, specific measures to prevent and control such unfortunate eventualities are essential (Boussinesq et al., 2001; Gardon et al., 1997). In the present trial, we avoided recruiting persons from communities with such high prevalence of *Loa* microfilaraemia.

Our trial was designed to evaluate the potential macrofilaricidal activity of ivermectin against *O. volvulus*, and the safety of normal and higher doses of ivermectin given at shorter intervals than in the regimen currently used as part of APOC. The examinations of nodules and skin snips after 3 years of repeated treatments led to two main results. The first was that 3-monthly treatments increase the mortality of adult female worms and have an increased sterilizing effect on those females that remain alive. Because of these effects, as well as the direct microfilaricidal action of ivermectin, 3-monthly treatments maintain the concentrations of mf in the skin at much lower levels than annual treatments. The second, and perhaps surprising, result was that high doses (800 μg/kg) produce little marginal parasitological benefit as compared with standard doses of 150 μg/kg. These observations lead us to recommend the use of ivermectin at 150 μg/kg 3-monthly rather than once a year.

Our results regarding adverse reactions reinforce the former conclusions, mainly based on parasitological arguments. First, throughout the 3 year trial, no severe adverse reaction was recorded; nor did we observe any case of orthostatic hypotension such as those reported from West Africa by Awadzi et al. (1990). The adverse reactions recorded during our trial were always transitory and responded readily to symptomatic treatment with analgaesics or antihistamines over 24–72 h. Only occasionally were corticosteroids necessary. Second, analysis

of the respective roles of dosage level and rhythm of treatment have demonstrated that taking ivermectin every 3 months is associated with a clearly reduced risk of adverse reactions, and that this decrease is particularly marked for the frequent and inconvenient reactions of oedema, itching and backache. Lastly, although we have shown that the dosage level seems to have little or no impact on the majority of reactions, two worrying manifestations, namely oedematous swellings and subjective ocular troubles, were more frequent after high doses than after the standard dose of 150 μg/kg.

In onchocerciasis the adverse reactions of ivermectin are linked to the death of mf in the skin or in the network of lymphatic capillaries, vessels and nodes, and to the resulting inflammatory reactions. Many studies have shown that the severity of such reactions is proportional to the degree of microfilaridermia (e.g. De Sole et al., 1989; Prod'hon et al., 1991), and that, under treatment, they tend to diminish year by year along with the *O. volvulus* microfilarial skin load. In our trial, this was particularly obvious in those groups treated with ivermectin at 3-monthly intervals.

One may wonder why, in our trial, the risk of occurrence of some reactions was related to the dose received, whereas others were not. The pathophysiology of post-ivermectin reactions is not fully understood, but is clearly associated with various immune responses (Njoo et al., 1993, 1994), including, particularly, eosinophil sequestration and activation/degranulation (Cooper et al., 1999, 2000). Recent results have also suggested that the *Wolbachia* bacterial endosymbiont of *O. volvulus* plays a role in mediating the inflammatory responses after treatment (Brattig et al., 2001; Keiser et al., 2002). In the present study, most of the symptoms seemed to occur at similar frequency, whatever the dose of ivermectin given. As indicated by Awadzi et al. (1995), this is probably because 'the Mazzotti reaction is a response to the death of microfilariae; and as near complete elimination is achieved with the standard dose, additional reaction is unlikely at higher doses'. However, we observed that the oedematous swellings were more frequent after high doses; this may possibly have resulted from the action of ivermectin on worms (probably males or possibly third or fourth-stage larvae or immature females) moving about outside the nodules. The issue of the higher frequency of subjective ocular symptoms after high doses will be addressed elsewhere, together with the results of two series of detailed ophthalmological examinations; but at present we have no explanation for these phenomena.

We have shown that high doses may be the cause of unexpected oedematous swellings, and especially of subjective ocular side reactions. In future, these findings should be taken into account if increasing the dose of ivermectin, to counteract any tendency of *O. volvulus* to develop a resistance to this drug, is considered. It should also be kept in mind, with regard to current trials evaluating the effects and safety of moxidectin, a close structural relative of ivermectin, but one, which has a considerably longer persistence in plasma.

All these results are consistent with our former conclusions (Gardon et al., 2002) that 3-monthly treatments with standard doses of ivermectin would have a great beneficial effect on communities suffering from onchocerciasis, and that this regimen should be further tested in the field to evaluate its feasibility under CDTI and its effect in reducing transmission of *O. volvulus*.

Ethical clearance
Ethical agreement to the trial was received from the Cameroonian Ministry of Public Health and from Merck & Co., Inc.

Conflicts of interest statement
The authors have no conflicts of interest concerning the work reported in this paper.

Acknowledgements

We thank Pr. Joseph Mbédé (former Minister of Public Health), who provided ethical clearance for this study; Dr Philippe Gaxotte (Merck & Co.) for his help in providing the capsules; and the patients who participated in the trial. The study was supported by the River Blindness Foundation.

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FOI 26-3132 - Document 5

Zinc: an essential but elusive nutrient[1–3]

Janet C King

ABSTRACT

Zinc is essential for multiple aspects of metabolism. Physiologic signs of zinc depletion are linked with diverse biochemical functions rather than with a specific function, which makes it difficult to identify biomarkers of zinc nutrition. Nutrients, such as zinc, that are required for general metabolism are called *type 2 nutrients*. Protein and magnesium are examples of other type 2 nutrients. Type 1 nutrients are required for one or more specific functions: examples include iron, vitamin A, iodine, folate, and copper. When dietary zinc is insufficient, a marked reduction in endogenous zinc loss occurs immediately to conserve the nutrient. If zinc balance is not reestablished, other metabolic adjustments occur to mobilize zinc from small body pools. The location of those pools is not known, but all cells probably have a small zinc reserve that includes zinc bound to metallothionein or zinc stored in the Golgi or in other organelles. Plasma zinc is also part of this small zinc pool that is vulnerable to insufficient intakes. Plasma zinc concentrations decline rapidly with severe deficiencies and more moderately with marginal depletion. Unfortunately, plasma zinc concentrations also decrease with a number of conditions (eg, infection, trauma, stress, steroid use, after a meal) due to a metabolic redistribution of zinc from the plasma to the tissues. This redistribution confounds the interpretation of low plasma zinc concentrations. Biomarkers of metabolic zinc redistribution are needed to determine whether this redistribution is the cause of a low plasma zinc rather than poor nutrition. Measures of metallothionein or cellular zinc transporters may fulfill that role. *Am J Clin Nutr* 2011;94(suppl):679S–84S.

INTRODUCTION

Zinc was shown to be an essential nutrient for rats and mice in the 1930s, for pigs in 1955, and for humans in 1963 (1). In the past 40 y, much has been learned about zinc's essential biochemical and physiologic functions. Although it is a dietary trace element, it is one of the most abundant elements within cells. Approximately 95% of the body zinc is within the cells. A complex, extensive system for cellular zinc homeostasis maintains cellular zinc concentrations within a narrow range.

Even though considerable progress has been made in understanding the regulation of cellular zinc metabolism, there still is much we do not know. The lack of a good indicator of zinc status is a primary limitation. The signs of zinc depletion are diverse and cannot be attributed to a defect in a specific function. Until now, efforts to identify a marker of zinc status have focused predominantly on the response to changes in dietary zinc intake. However, studies of cellular zinc metabolism show that shifts in zinc-dependent functions are frequently linked to intracellular zinc fluctuations. This suggests that cellular zinc metabolism needs to be assessed along with whole-body zinc homeostasis to fully understand zinc status.

This article is divided into 3 parts: an overview of zinc's essential functions, a discussion of *why* assessing zinc status is so elusive, and a summary of cellular and whole-body potential biomarkers of zinc nutrition. Excessively high zinc intakes undoubtedly also alter these biomarkers, but given the limited information on the zinc metabolic response to excessive intakes, that aspect is not addressed in this article.

ZINC ESSENTIALITY

The physiologic effects of zinc deficiency are associated with a number of diverse biochemical changes rather than with a specific function as is the case for other trace elements such as iron and selenium. In general, zinc is essential for normal metabolism. Given its ubiquitous nature and high intracellular concentrations, it is not surprising that zinc has 3 very basic functions: catalytic, structural, and regulatory (2).

Zinc is a catalyst for >50 different enzymes when the same enzyme from different sources (plant, animal, microbial) is counted only once (2). An enzyme is classified as a zinc metalloenzyme if removal of zinc causes a decrease in activity without affecting the protein and if adding back zinc restores enzyme activity. A direct link between zinc deficiency symptoms and the function of an individual enzyme or enzymes has not been identified in complex organisms. Such a direct link is unlikely because it would occur only if the zinc-dependent enzyme were acting at a rate-limiting step in a critical biochemical pathway. For example, alkaline phosphatase is a zinc-dependent enzyme whose activities decline with low zinc intakes. But, it has not been possible to relate the signs and symptoms of zinc depletion with a change in alkaline phosphatase activity.

The discovery of the zinc finger motif in frogs in 1985 established a structural role for zinc (2). Zinc fingers have 4 cysteines within the general structure of the protein that allows zinc to be bound in a tetrahedral complex. Some zinc fingers have histidine substituted for cysteine. Zinc fingers occur in a variety

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First published online June 29, 2011; doi: 10.3945/ajcn.110.005744.

FOI 26-3132 - Document 5

of proteins, including those involved in cellular differentiation or proliferation, signal transduction, cellular adhesion, or transcription. Zinc is also involved in maintaining the structure of enzymes such as CuZn superoxide dismutase where copper is at the active site and zinc maintains enzymatic structure. The extent to which dietary zinc intake influences the function of zinc finger proteins is not well known. However, given the abundance of zinc fingers, these structures contribute to the overall zinc requirement and provide a rationale for the tight homeostatic control of zinc metabolism.

The third basic zinc function is regulation of gene expression (2). The basic components include a metal-binding transcription factor (MTF) and a metal response element (MRE) in the promoter of the regulated gene. The MTF acquires zinc in the cytosol or nucleus and then interacts with the MRE to stimulate transcription. MTF-1 is thought to regulate numerous genes either negatively or positively depending on the cellular zinc status (2). It is thought that dietary zinc can be transported into the cells and interact with MTF-1, facilitating translocation to the nucleus for MRE binding and stimulating transcription. This may be the mechanism whereby adaptive changes in lipid peroxidation, apoptosis, immunity, and neuronal function occur with zinc depletion.

These general zinc biochemical functions have not been related to the physiologic effects of zinc depletion. This is because these basic biochemical zinc functions do not provide a complete picture of cellular zinc metabolism and function. Zinc transporters also play a key role in moving zinc to various organelles, thereby regulating cellular function. For example, the secretion of neurotransmitters, such as glutamate from neuronal synaptic vesicles, requires zinc and explains the relation between zinc and cognitive function (3). Also, zinc is required for packaging insulin into pancreatic granules and its subsequent secretion. Thus, zinc transporters also contribute to metabolic regulation by moving zinc into and out of organelles, thereby influencing gene transcription or the secretion of essential hormones or neurotransmitters.

Two classes of zinc transporters have been identified (2, 3). The ZIP (SLC 39) family of transporters move zinc into the cytoplasm from extracellular space or cellular organelles. A total of 14 ZIP transporters have been identified. The ZnT (SLC 30) family of transporters move zinc and other metal ions, such as iron, from the cytoplasm into the lumen of the intracellular organelles or to the outside of the cell. Thus, the ZnT proteins work in opposition to the ZIP transporters. A total of 10 ZnT transporters have been identified (2, 3). Examples include ZnT3, which is found in the synaptic vesicles of neurons, and ZnT8, which is found in the secretory granules of pancreatic β cells. Zinc transporters play a major role in cellular zinc trafficking, thereby regulating zinc function. Although the discovery of zinc transporters has expanded our understanding of zinc metabolism, much remains to be learned. For example, what do all of the 24 zinc transporters do and how is their expression altered to help maintain cellular zinc homeostasis?

WHY IS ZINC STATUS ELUSIVE?

Most nutrients are required for specific metabolic functions rather than for metabolism in general as is the case for zinc (4). When a deficiency of nutrients with specific functions occurs, tissue nutrient concentrations decline and a defect in one or more specific metabolic pathways develop, causing the onset of specific clinical or biochemical signs. The diagnosis of a deficiency of nutrients with specific functions is relatively straightforward. All one has to do is measure the nutrient concentration in an appropriate, convenient tissue; test the metabolic pathways where the defect lies; or simply recognize the specific clinical sign. Nutrients with specific functions are referred to as "type 1 nutrients." Examples of specific indicators of type 1 nutrient deficiencies include microcytic anemia for iron, beriberi for thiamine, pellagra for niacin, scurvy for vitamin C, and macrocytic anemia for folic acid.

Type 2 nutrients are required for multiple general metabolic functions and therefore respond to insufficient intakes quite differently (4). Examples include nitrogen, essential amino acids, magnesium, potassium, and zinc. The first response to an insufficient intake of type 2 nutrients is a marked reduction in endogenous losses and possibly a metabolic adaptation to reduce the need for functions that have a high demand, such as growth or immunity. For example, if a type 2 nutrient deficiency occurs in a growing animal, a marked decline in growth occurs within days, which is soon followed by nonspecific signs of metabolic or clinical dysfunction. General dysfunction—ie, malaise or apathy—is rapid because there is no body store or reserve of type 2 nutrients that lasts for more than a couple of days in growing animals.

Zinc is a classic example of a type 2 nutrient. An inadequate zinc intake in experimental animals or humans causes an avid reduction of excretory losses to conserve zinc (5). Endogenous fecal losses decrease markedly within a couple days. With severe zinc depletion ($<$1 mg dietary zinc/d), urinary losses also decline (6). If the decline in fecal and urinary losses fails to reestablish zinc balance, additional metabolic adjustments occur to mobilize zinc from a small, vulnerable pool for zinc function. Tissue catabolism may also occur to release zinc. Experimental animals fed diets providing $<$3 parts per million (ppm) zinc develop a cyclical food intake pattern in which anorexia and food ingestion fluctuate over a 4–5-d period (7). During the anorexic period, muscle is catabolized, and zinc along with protein, potassium, and other muscle components are released. Flanagan (8) showed that cyclic food intake permits longer survival in rats fed a zinc-deficient diet. Rats tube-fed a zinc-deficient diet thrived for 6–7 d and then became seriously ill, whereas rats fed the deficient diet ad libitum and that then underwent cyclical food intakes stopped growing after 3 d, but they remained relatively healthy for $\approx$10 d.

Because zinc losses are markedly reduced with severe depletion, the total amount of zinc lost is small. For example, men subsisting on a virtually zinc-free diet for 4–9 wk lost a total of $\approx$95 mg ($\approx$1460 μmol) zinc, which represents $\approx$5% of total body zinc (5). In a 5-wk severe-zinc-depletion study, zinc losses totaled $\approx$40 mg ($\approx$615 μmol), or $\approx$2% of body zinc (9). Clinical symptoms of zinc depletion—ie, skin lesions, diarrhea, and sore throats—were evident in both studies. This shows that the vulnerable zinc pool is very small, and it is sufficient only to support body needs for several weeks when the diet lacks zinc. The corollary of this small zinc reserve is that the response to zinc repletion is extremely rapid. Within a matter of days, the clinical picture changes: enzymes are resynthesized (7), a growth response is observed in children (10), and skin lesions are reversed (11). This rapid reversal of clinical symptoms with small

FOI 26-3132 - Document 5

increases in dietary zinc occurs because there is no significant loss of tissue zinc with depletion. There is no large depleted pool that must be replaced before clinical symptoms improve. Instead, only enough is needed to replete a very small, vulnerable pool (4).

It is important to remember that all nutrients excreted as a consequence of muscle catabolism due to zinc deficiency must be replaced during the repletion period. If, for example, an anorexic child with zinc deficiency is supplemented with zinc, he will regain his appetite and, if quality foods are available, his intake of protein, potassium, and intrinsic dietary zinc will increase. However, if the diet staple is a low-quality cereal food, he may not consume sufficient amounts of the other type 2 nutrients lost and the growth response may be limited. The growth response of stunted children given supplemental zinc varies widely in studies done around the world (12). This may reflect an insufficient intake of other type 2 nutrients, such as protein and potassium, during the zinc supplementation trial.

POTENTIAL DIAGNOSTIC BIOMARKERS OF ZINC NUTRITION

Unique features of type 2 nutrients

Aggett (13) defined nutritional status as the ratio of the tissue nutrient need to tissue nutrient availability. If need is greater than availability and the ratio is less than one, nutritional status is inadequate. This definition assumes that a decline in nutritional status is associated with a reduction in tissue nutrient concentration. This is true for type 1 nutrients where a low tissue nutrient concentration causes a decline in one or more specific functions (4). Type 2 nutrients respond differently to dietary insufficiency because their tissue pools are fixed and vary little with reduced intakes. Instead, a small, vulnerable body pool exists that must be sustained by a continual dietary supply. When intakes are insufficient for maintaining balance, this small pool is depleted quickly, often within days in growing animals or weeks in adult humans, giving rise to nonspecific symptoms of nutrient depletion, such as reduced growth, skin lesions, or infection. Because zinc is a type 2 nutrient, the response to zinc depletion and repletion is extremely rapid (4).

Homeostatic response to acute zinc depletion

Studies in animals and humans show that symptoms of severe zinc depletion occur rapidly and recover quickly with repletion (5, 7, 9, 14). For example, growth ceased after 4 d of a zinc-deficient diet (≈1 ppm) in weanling rats (7). In children, a rapid growth response was observed during zinc repletion (15). Also, the onset of clinical signs of zinc depletion occurred rapidly in adult men who consumed zinc-deficient diets (16, 17). A description of the clinical lesions observed in a study of 8 men who consumed a diet virtually free of zinc, the time of onset, and plasma zinc concentration at that time are shown in **Table 1** (17). Only one subject (12) had no health problems, and subject 5 developed only a sore throat without any skin lesions. The plasma zinc concentrations of these 2 men at the end of depletion (day 35 or 36) tended to be higher than that of the other men, ≈49 μg/dL. Three subjects (subjects 2, 3, and 5) complained of sore throats on day 26 of depletion. Toward the end of depletion (day 33 or 40), 6 subjects (subjects 1–4, 10, 11) had developed canker sores of the mouth and nostrils and a rash on their faces. Subjects 1 and 11 also had rashes in the scrotal area. Plasma zinc concentrations had dropped below 35 μg/dL when the skin rashes occurred. After only 6 d of repletion with 12.2 mg zinc/d, all symptoms had vanished. When the first sign of skin lesions occurred (day 28), whole-body zinc losses totaled 42 mg (≈645 μmol or ≈2–3% of whole-body zinc); at the end of depletion, after ≈40 d, total losses averaged 45 mg, showing the marked effect of the decline in excretory losses on total body zinc loss during extended time on the poor diet (16). It appears, therefore, that the vulnerable pool mobilized with zinc deficiency before clinical signs of zinc depletion are evident is <2% of body zinc. Given this limited reserve of only ≈40 mg zinc to maintain function when the diet is insufficient, it is not surprising that zinc endogenous losses rapidly approach zero in an attempt to maintain the pool and tissue zinc homeostasis with a marked reduction in intake.

TABLE 1
Time of appearance of clinical signs of zinc depletion and plasma zinc concentrations

Subject no.	Day of depletion	Plasma zinc	Clinical signs
		μg/dL	
1	32	8.0	Rash on face, canker sores in mouth, rash in scrotal region, fungal infection on feet
2	26	22.2	Sore throat
	32	17.2	Mouth canker sores, facial rash
3	25	<59.7[1]	Sore throat
	34	19.1	Mouth and nasal canker sores
4	37	24.0	Mouth canker sores
	40	15.8	Facial rash with purulent drainage
5	26	<49.0[1]	Sore throat
	35	48.7	Sore throat
10	34	35.5	Mouth canker sores
11	36	27.6	Nasal canker sores
			Rash in scrotal region, lesion on penis
12	36	48.6	No problems
	40	39.7	No problems

[1] Plasma zinc was not measured on this day. The value given is the previous measurement.

FOI 26-3132 - Document 5

Zinc pools sensitive to zinc deficiency

Cellular organelles

The location or locations of the small, vulnerable zinc pool sensitive to zinc depletion has not been established. Components of the small, vulnerable zinc pools are probably found in all tissues (3, 15). In yeast, which does not have zinc efflux systems, excessive zinc is transported into intracellular organelles for storage and release in times of need. This storage organelle is called a zincosome (3). In mammalian cells, it appears that a small reserve of zinc is stored in the Golgi and in the endoplasmic reticulum. To maintain cellular zinc homeostasis, the ZnT (SLC30) transporters efflux zinc into those organelles or across the plasma membrane (3).

Cellular metallothionein

Cellular metallothionein is another means whereby cells can "park" zinc temporarily for future needs (4). Metallothionein is a cysteine-rich, intracellular, metal-binding protein (18). Although metallothionein has a higher affinity for copper and cadmium than for zinc, it normally exists in vivo in the zinc form. Up to 7 zinc molecules can bind to one metallothionein molecule. Four major metallothionein isoforms exist: MT-1 and MT-2 are located throughout the body, MT-3 is found primarily in the brain, and MT-4 is most abundant in stratified tissues (19). MT-2a is the most predominant form in human tissues. The highest concentrations of metallothionein are found in the liver, kidney, intestine, and pancreas (18). Gut and pancreatic metallothionein concentrations respond readily to changes in dietary zinc, suggesting that metallothionein helps maintain zinc homeostasis in these tissues. For example, high concentrations of intestinal zinc induce metallothionein synthesis and limit zinc absorption by sequestering it in the intestinal wall. The role of metallothionein in enhancing zinc absorption with low zinc intakes is uncertain. However, a decline in pancreatic and renal metallothionein occurs with low zinc intakes and facilitates the decline in fecal and urinary zinc losses (18).

Liver metallothionein concentrations respond promptly to tissue injury, infection, inflammation or neoplastic disease (18, 20, 21). In an inflammatory state, hepatic metallothionein content can increase as much as 100-fold within 2–4 h. This sharp rise in hepatic metallothionein causes hepatic uptake of plasma zinc to facilitate the acute stress response associated with inflammation.

Although the largest concentrations of metallothionein are in the liver, kidney, intestine, and pancreas, smaller quantities are located in all tissue cells. Because metallothionein gene expression is regulated by zinc and because it is one of the strongest cellular zinc binding proteins, it is likely that metallothionein also provides a way for cells to maintain a small zinc reserve (2, 18, 20, 21). Furthermore, the release of zinc from metallothionein when needed causes metallothionein to be degraded, freeing the zinc for metabolic functions.

Plasma zinc

Plasma zinc is a pool of zinc readily available for uptake by tissues when needed, and it is a component of the small, vulnerable zinc reserve. Studies in men show that plasma zinc mass declines rapidly with severe deficiency, decliningfrom 3.4 mg to 1.2 mg in 5 wk (9). The important role of plasma zinc, which serves as a source of zinc for essential tissue functions with low intakes, is shown by its high fractional turnover rate. Normally, the total amount of zinc in plasma is replaced ≈150 times/d; this increases to ≈200 times/d with severe zinc deficiency (9). The decline in plasma zinc concentrations during severe depletion is highly correlated with zinc balance; the higher the loss of body zinc, the greater the rate of plasma zinc decline (22). With moderate dietary zinc restriction (3–5 mg/d), the plasma zinc response is subtle, and shows no change to a slight decrease in zinc because the amount and rate of whole-body zinc loss is lower (23). A comprehensive evaluation of the relation between dietary zinc and plasma zinc concentrations shows that plasma concentrations decrease sharply when intakes are <2–3 mg/d but rise slightly and continuously with higher intakes, reaching a peak at ≈25–30 mg/d (23). It is interesting that zinc absorption also seems to plateau at intakes >20 mg/d. Supplementation with 10 or 20 mg zinc in healthy adult men increased plasma zinc concentrations within 5 d, but concentrations also declined to baseline levels within 5 d of discontinuing zinc supplementation (24).

Although plasma zinc concentrations are sensitive to zinc nutrition, they is not a specific measure of zinc nutrition. This is because plasma zinc is mobilized as a result of metabolic redistribution as well as poor zinc nutrition. Plasma zinc readily decreases with metabolic redistribution induced by endotoxemia, infection, carcinoma, steroid use, and after a meal (4, 25). This decline represents a redistribution of zinc from the small, vulnerable pool to the tissues, not a change in zinc nutrition.

Plasma zinc is primarily bound to albumin (70%) with the remainder bound tightly to α_2-macroglobulin (18%) and other proteins or amino acids, especially histidine and cysteine (23). Hypoalbuminemia is frequently associated with hypozincemia (4), and it is argued that this is due to reduced concentrations of plasma zinc–binding sites that could confound the interpretation of the decline in plasma zinc concentrations. However, even when plasma albumin is low, only ≈1 of every 50 albumin molecules is associated with a zinc atom, making it difficult to see how a reduction in albumin could lead to a significant reduction in zinc (4). On the other hand, a marginal zinc status could lead to a reduction in albumin synthesis.

Food intake, plasma volume expansion (pregnancy, overhydration), and steroid or oral contraceptive use all cause a decline in plasma zinc concentrations. A careful assessment of the individual's health status at the time of the blood draw can help determine whether the plasma zinc concentration is low due to one of these conditions. Ruling out the influence of a subclinical infection as a cause of low plasma zinc concentrations is more difficult. Measurements of proteins that are elevated in response to tissue injury or infection—eg, C-reactive protein or α_1-acid glycoprotein—are frequently performed to determine whether infection is present (26). However, these measures of infection are not directly related to plasma zinc concentrations because the stage and severity of infection both influence the change in plasma zinc. Also, the nutritional status of the host will affect the influence of infection on plasma zinc concentrations. The cytokine response to infection is reduced in malnourished animals and humans, suggesting that infection may have a smaller effect on plasma zinc concentrations in malnourished individuals (26).

FOI 26-3132 - Document 5

Theoretically, metallothionein may be the key to diagnosing metabolic zinc redistribution due to infection, trauma, or stress (4). Metallothionein can be detected in plasma and erythrocytes by a radioimmunoassay (27, 28). Hepatic metallothionein concentrations rise with inflammation or stress, which also causes a rise in plasma metallothionein but not erythrocyte metallothionein (20, 21). Thus, in a metabolic state causing tissue zinc redistribution, plasma zinc concentrations will be low and metallothionein concentrations will be high, but erythrocyte metallothionein will be normal (16). If the plasma zinc pool is reduced due to poor zinc nutrition in the absence of infection, both plasma and erythrocyte metallothionein should be low.

This potential procedure for determining if low plasma zinc is due to poor nutrition or metabolic distribution has not been validated experimentally. But, preliminary studies are promising. Human studies have shown that both plasma and erythrocyte metallothionein concentrations are sensitive to dietary zinc (21, 27). However, the effects of infection or stress on plasma and erythrocyte metallothionein concentrations in the presence of low or adequate zinc nutrition need to be evaluated in humans.

Exchangeable zinc pool

Using zinc isotopes and a model-based compartmental analysis, Miller et al (29) and Jackson et al (30) have defined an exchangeable zinc pool (EZP) that exchanges rapidly with plasma zinc. Some, but not all, studies have shown a relation between dietary zinc and the size of the EZP (31, 32). During severe zinc depletion in adult men, EZP size declined nearly as much as the plasma zinc (60% compared with 65%), suggesting that it is part of the small, vulnerable pool susceptible to zinc depletion but not more sensitive than plasma zinc concentrations. Measurements of the effect of infection or stress on the size of the EZP have not been done. But it is likely that EZP size will decline with metabolic zinc redistribution due to stress because plasma zinc is a component of the EZP measure. Presently, EZP size is not used widely as a biomarker of zinc nutrition because it is not more responsive to changes in dietary zinc than is plasma zinc and because its measurement requires the use of expensive stable isotopes and access to a mass spectrometer for analysis.

CONCLUSIONS

Although much has been learned about cellular and whole-body zinc homeostasis and metabolism, a sensitive, specific biomarker of zinc nutrition has not been identified. Given that zinc is required for multiple general metabolic functions, it is unlikely that a specific biomarker of zinc nutrition exists. Nevertheless, the sensitivity of a small, vulnerable zinc pool to dietary zinc provides a means of identifying zinc under- and possibly overnutrition. Plasma zinc is a component of the small, vulnerable zinc pool, and it is a sensitive marker of zinc nutrition. Unfortunately, plasma zinc concentration is subject to metabolic redistribution as well as insufficient dietary zinc. Research is needed to identify biomarkers of zinc metabolic redistribution. Indicators of shifts in the metabolism of cellular zinc pools (zinc transporters or metallothionein) appear to be promising.

The author did not declare any conflicts of interest.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Doxycycline Inhibits TGF-β1–Induced MMP-9 via Smad and MAPK Pathways in Human Corneal Epithelial Cells

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Purpose. To evaluate the effects of TGF-β1 and doxycycline on production of gelatinase MMP-9 and activation of Smad, c-Jun N-terminal kinase (JNK), extracellular-regulated kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) signaling pathways in human corneal epithelial cells.

Methods. Primary human corneal epithelial cells were cultured to confluence. The cells were treated with different concentrations of TGF-β1 (0.1, 1, or 10 ng/mL), with or without TGF-β1–neutralizing mAb (5 μg/mL), SP600125 (30 μM), PD98059 (40 μM), SB202190 (20 μM), or doxycycline (5–40 μg/mL) for different lengths of time. Conditioned media were collected from cultures treated for 24 to 48 hours to evaluate the MMP-9 production by zymography and activity assay. Total RNA was isolated from cells treated for 6 to 24 hours to evaluate MMP-9 expression by semiquantitative RT-PCR and Northern hybridization. Cells treated for 5 to 60 minutes were lysed in RIPA buffer for Western blot with phospho-specific antibodies against Smad2, JNK1/2, ERK1/2, or p38.

Results. TGF-β1 increased expression, production, and activity of MMP-9 by human corneal epithelial cells in a concentration-dependent fashion. TGF-β1 also induced activation of Smad2, JNK1/2, ERK1/2, and p38 within 5 to 15 minutes, with peak activation at 15 to 60 minutes. Doxycycline markedly inhibited the TGF-β1–induced production of MMP-9 and activation of the Smad, JNK1/2, ERK1/2, and p38 signaling pathways. Its inhibitory effects were of a magnitude similar to SP600125, PD98059, and SB202190, specific inhibitors of the JNK1/2, ERK1/2, and p38 pathways, respectively.

Conclusions. These findings demonstrated that doxycycline inhibits TGF-β1–induced MMP-9 production and activity, perhaps through the Smad and MAPK signaling pathways. These inhibitory effects may explain the reported efficacy of doxycycline in treating MMP-9–mediated ocular surface diseases. (*Invest Ophthalmol Vis Sci.* 2005;46:840–848) DOI:10.1167/iovs.04-0929

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Presented at the annual meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, Florida, May 2003.

Supported part by National Eye Institute Grants EY11915 (SCP) and EY014553 (D-QL), an unrestricted Grant from Research to Prevent Blindness, the Oshman Foundation, and the William Stamps Farish Fund.

Submitted for publication August 2, 2004; revised October 11, 2004; accepted November 23, 2004.

Disclosure: **H.-S. Kim**, None; **L. Luo**, None; **S.C. Pflugfelder**, None; **D.-Q. Li**, None

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The important role of matrix metalloproteinases (MMPs) has been recognized in ocular surface diseases including wound healing, dry eye, sterile corneal ulceration, recurrent epithelial erosion, corneal neovascularization, pterygium, and conjunctivochalasis.[1–6] Among these, gelatinase B (MMP-9) has been found to be of central importance in extracellular matrix remodeling after wounding of the corneal surface and has been implicated in the pathogenesis of sterile corneal ulceration, dry eye–associated epitheliopathy, and other ocular diseases.[7–10] The expression of MMP-9 is known to be modulated by cytokines and growth factors that induce cellular responses by activating a variety of intracellular signaling cascades. The pro-inflammatory cytokines IL-1β and TNF-α, as well as transforming growth factor (TGF)-β have been reported to play a role in the regulation of MMP-9 production and activity in human corneal epithelial cells.[11]

TGF-β is known to play a vital role in tissue remodeling during inflammatory responses. TGF-β has been found to stimulate the expression of MMP-9 in endothelial cells, fibroblasts, and epithelial cells, including the corneal epithelium.[11–14] The diverse cellular responses elicited by TGF-β are triggered by activation of serine/threonine kinase TGF-β receptors and are transduced through multiple intracellular signal pathways. After ligand binding, the type II TGF-β receptor recruits the type I receptor, which is then phosphorylated by the type II receptor. The activation of the type I receptor triggers the phosphorylation of cytoplasmic proteins, including the novel Smad pathway, that are directly implicated in the transmission of the intracellular signal.[15] Many of the signaling responses induced by TGF-β are mediated by Smad proteins, but certain evidence has suggested that TGF-β can also signal independently of Smads.[16] TGF-β can activate mitogen-activated protein kinase (MAPK) signaling pathways including c-Jun N-terminal kinase (JNK), extracellular-regulated kinase (ERK), and p38 MAPK, and the specific pathway used appears to vary with and depend on the cell type and tissue function.[17,18] The signaling pathways that mediate the regulatory effect of TGF-β on MMP-9 are not understood.

Doxycycline, a long-acting semisynthetic tetracycline, is well recognized for its therapeutic efficacy in treating MMP-mediated ocular surface diseases, such as rosacea, and sterile corneal ulceration.[19,20] Doxycycline has been found to inhibit MMP-9 activity in human endothelial cells,[21] skin keratinocytes,[22] and cancer cells.[23,24] Doxycycline was reported to significantly decrease serum MMP-9 levels (50%) after 6 months of oral administration after myocardial infarction.[25] Doxycycline has been reported to decrease the activity of MMP-9 in conditioned media of cultured human corneal epithelial cells,[26] but the effects and mechanisms of doxycycline on TGF-β–induced MMP-9 production are not known.

This study investigated the effects of doxycycline on TGF-β1–induced production of MMP-9 and activation of the Smad2, JNK, ERK, and p38 MAPK signaling pathways in human corneal epithelial cells.

Materials and Methods

DMEM/Ham's F12, amphotericin B, phenol, DNA or RNA size markers, and a random primer DNA labeling kit were purchased from Invitro-

Investigative Ophthalmology & Visual Science, March 2005, Vol. 46, No. 3
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gen-Gibco (Grand Island, NY); fetal bovine serum (FBS) from Hyclone (Logan, UT); recombinant human TGF-β1 and TGF-β1 neutralizing monoclonal antibody (mAb) from R&D Systems (Minneapolis, MN); activity assay kits (Biotrak) for MMP-9 and chemiluminescence reagents (ECL Plus) from Amersham Pharmacia Biotech (Piscataway, NJ); EDTA-free protease inhibitor cocktail tablet from Roche Applied Sciences (Indianapolis, IN); the bicinchoninic (BCA) protein assay kit from Pierce Chemical (Rockford, IL); SP600125, PD98059, and SB202190 from Calbiochem (Darmstadt, Germany); rabbit polyclonal antibodies against ERK or p38 from Cell Signaling (Beverly, MA); phospho-specific mAbs for JNK, ERK, and p38 and rabbit antibodies against JNK and phospho-Smad2 from Santa Cruz Biotechnology (Santa Cruz, CA); polyvinylidene difluoride (PVDF) membranes from Millipore (Bedford, MA); nitrocellulose membranes from Schleicher and Schuell (Keene, NH); the PCR kit (GeneAmp) from Applied Biosystems (Foster City, CA); PCR purification and gel extraction kits (QIAquick) from Qiagen (Valencia, CA); [α-^{32}P]-dCTP from Du Pont NEN (Boston, MA); and film and intensifying screens (XAR-5 and BioMax MS-1) from Eastman Kodak (Rochester, NY). All plastic ware was from BD Biosciences (Franklin Lakes, NJ). Doxycycline, cholera toxin subunit A, hydrocortisone, and all other reagents were from Sigma-Aldrich (St. Louis, MO).

Primary Cultures of Human Corneal Epithelial Cells

Human corneal epithelial cells were cultured from explants taken from human donor corneoscleral rims, provided by the Lions Eye Bank of Texas, using a previously described method.[26,27] In brief, corneoscleral rims were trimmed, the endothelial layer and iris remnants were removed, and each limbal rim was dissected into 12 equal segments. Two segments were placed in each well of six-well culture plates, and each explant was covered with a drop of FBS overnight. The explants were then cultured in hormone supplemented medium, containing equal amounts of DMEM and Ham's F12, supplemented with 5% FBS, 0.5% dimethyl sulfoxide, 2 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 0.5 μg/mL hydrocortisone, 30 ng/mL cholera toxin A, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B, at 37°C in 95% humidity and 5% CO_2. The medium was renewed every 2 to 3 days. Epithelial phenotype of these cultures was confirmed by characteristic morphology and immunofluorescent staining with cytokeratin antibodies (AE-1/AE-3). Subconfluent cultures (12–14 days) were washed four times with PBS and switched to serum-free medium (the same just medium described, but without FBS) for 24 hours before treatment.

Cell Treatment

Except for the control groups that were continually cultured in serum-free medium alone, the cultures were treated with TGF-β1 at different concentrations (0.1, 1.0, or 10 ng/mL), with or without the TGF-β1-neutralizing mAb (5 μg/mL), SP600125 (30 μM, a JNK/SAPK pathway inhibitor), PD98059 (40 μM, a ERK MAPK pathway inhibitor), SB202190 (20 μM, a p38 MAPK pathway inhibitor), or doxycycline (5–40 μg/mL) for different lengths of time. For gelatin zymography and MMP-9 activity assays, the same volume of the serum-free medium (1.2 mL) was added to each well of corneal epithelial cultures (~5×10^5 cells/well). The conditioned medium after 24 to 48 hours of treatment was collected and centrifuged, and the supernatants were stored at −80°C before use. The adherent cells were lysed in phosphate-buffered saline (PBS, pH 7.3), containing 1.5 M NaCl and 0.039% Triton X-100 for the BCA protein assay. The cellular protein concentration in each well was used to adjust the volume of the conditioned medium used for gelatin zymography and MMP-9 activity assays. For MMP-9 gene expression analysis, the corneal epithelial cells after treatment for 6 hours were lysed in 4 M guanidium solution and subjected to total RNA extraction. The cells treated for shorter times (5, 15, 30, and 60 minutes) were lysed in RIPA lysis buffer, containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS, and an EDTA-free protease inhibitor cocktail tablet, for Western blot analysis. These experiments were performed at least three times using separate sets of cultures that were initiated from different donor corneas.

Gelatin Zymography

To determine the relative concentration and activity of gelatinases in the conditioned media from corneal epithelial cultures receiving various treatments, SDS-PAGE gelatin zymography was performed with a previously reported method.[28] Briefly, 8 to 10 μL of each conditioned medium was used, and the volume was adjusted by cellular protein concentrations. The media supernatants were treated with SDS-PAGE sample buffer without boiling or reduction. Samples were fractionated in an 8% polyacrylamide gel containing gelatin (0.5 mg/mL) by electrophoresis at 100 V for 90 minutes at 4°C. The gels were soaked in 0.25% Triton X-100 for 30 minutes at room temperature to remove the SDS, and incubated in a digestion buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10 mM $CaCl_2$, 2 μM $ZnSO_4$ and 0.01% Brij-35) containing 5 mM phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, at 37°C overnight, to allow proteinase digestion of its substrate. The gels were then stained with 0.25% Coomassie brilliant blue R-250 in 40% isopropanol for 2 hours, and destained with 7% acetic acid. Gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

MMP-9 Activity Assay

The total activity levels of MMP-9 protein in supernatants of the corneal epithelial cultures were determined using MMP-9 activity assay systems (Biotrak) according to manufacturer's protocol. This assay primarily measured the activity of latent MMP-9, because the active form of MMP-9 in the media was almost undetectable. In brief, 100 μL each pro-MMP-9 standard (0.5–32 ng/mL), culture supernatant sample, or assay buffer (for blanks) was incubated at 4°C overnight in microtiter wells precoated with anti-MMP-9 antibody. Any MMP-9 present in these solutions bound to the wells. Other components of the sample were removed by washing four times with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.05% Tween-20. To measure the total activity of MMP-9, bound pro-MMP-9 was activated with 50 μL of 1 mM *p*-aminophenylmercuric acetate (APMA) in assay buffer at 37°C for 2 hours. Detection reagent (50 μL) was then added to each well and incubated at 37°C for 6 hours. Active MMP-9 was detected though activation of a modified prodetection enzyme and the subsequent cleavage of its chromogenic peptide substrate. The resultant color was read at 405 nm in a microtiter plate reader. The activity of MMP-9 in a sample was determined by interpolation from a standard curve.

RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from corneal epithelial cell cultures by acid guanidium thiocyanate-phenol-chloroform extraction according to a previously described method.[29] The PCR primers for MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed from published human gene sequences (Table 1). The semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed to evaluate the expression of these MMP-9 genes by corneal epithelial cells with a housekeeping gene, GAPDH, as an internal control.[11] In brief, the first-strand cDNAs were synthesized from 1 μg of total RNA at 42°C for 30 minutes. PCR amplification was performed in a DNA thermal cycler (model 9700; Applied Biosystems, Inc.) using the following program: denaturation for 2 minutes at 95°C followed by 20 to 40 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 60°C, and extension for 1 minute at 72°C, with a final extension for 7 minutes at 72°C. Semiquantitative RT-PCR was established by terminating reactions at intervals of 20, 24, 28, 32, 36, and 40 cycles for each primer pair to ensure that the PCR products formed were within the linear portion of the amplification curve. The fidelity of the RT-PCR product was verified by comparing the size of the

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TABLE 1. Human Primer Sequences Used for RT-PCR and Probe Preparation

Gene	Accession No.	Sense Primer	Antisense Primer	Probe (bp)
MMP-9	NM_004994	ATCCAGTTTGGTGTCGCGGAGC	GAAGGGGAAGACGCACAGCT	552
GAPDH	M33197	GCCAAGGTCATCCATGACAAC	GTCCACCACCCTGTTGCTGTA	498

amplified products to the expected cDNA bands and by sequencing the PCR products.

Probe Preparation and Northern Hybridization

Human cDNA probes (552-bp fragment of MMP-9 and 498 bp of GAPDH) were purified from the RT-PCR products by electrophoresis through a 1.5% low-melting-point agarose gel, with a PCR purification kit (QIAquick; Qiagen) and gel extraction kit, per the manufacturer's protocol. Northern hybridization was performed with a previously described method.[29] In brief, total RNA for each group at 20 μg/lane was electrophoresed through 1.2% agarose containing formaldehyde, transferred to nitrocellulose membranes, and hybridized with a ^{32}P-labeled cDNA probe at 2 to 4 $\times$ 10^6 cpm/3 to 8 ng/mL in the hybridization solution. After visualizing the hybridization product on the x-ray film, the ^{32}P-label on the membrane was stripped by washing the membranes twice at 65°C for 1 hour in 5 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.05% sodium pyrophosphate, and 0.1$\times$ Denhardt's solution. The membranes were then rehybridized with GAPDH probe, which served as a loading control.

Western Blot Analysis

The cells treated for 5 to 60 minutes were lysed in RIPA buffer, and appropriate volumes (25–30 μL) of cell extracts, adjusted to represent the same amount of total cellular protein (50 μg), were electrophoresed under reducing condition at 4°C in a 4% to 15% gradient polyacrylamide gel. After electrophoretic transfer to a PVDF membrane at 100 V for 1 hour at 4°C, the membranes were blocked with 5% nonfat milk in TTBS (50 mM Tris [pH 7.5], 0.9% NaCl, and 0.1% Tween-20) for 1 hour at RT. The primary antibody (i.e., phospho-specific antibodies against Smad2, JNKs, ERKs, or p38) in TTBS containing 5% nonfat milk was placed on each membrane and incubated at RT for 60 minutes with agitation. After three washings with TTBS over 15 minutes, the second antibody, conjugated with horseradish peroxidase (HRP), was applied and the signals on the membranes were detected by chemiluminescence reagents (ECL Plus; Amersham Pharmacia Biotech) and imaged with a digital image station (2000R; Eastman Kodak).

RESULTS

Effects of TGF-β1 and Doxycycline on MMP-9 Production and Activity

The protein levels of MMP-9 were determined by gelatin zymography and MMP-9 activity assay in the serum-free conditioned medium from corneal epithelial cultures. As shown in Figures 1A and 1B, zymography revealed that TGF-β1 stimulated 92-kDa MMP-9 protein production in a concentration-dependent manner by corneal epithelial cells treated with increasing concentrations of TGF-β1 (0.1, 1.0, and 10 ng/mL) for 24 hours. TGF-β1 at 1 ng/mL markedly stimulated MMP-9 production and the strongest stimulation was observed with 10 ng/mL. This stimulation at 1 ng/mL TGF-β1 was abolished by 5 μg/mL TGF-β1 neutralizing mAb or 10 μg/mL doxycycline (Fig. 1A). In contrast, the bands of 72-kDa gelatinase A (MMP-2) were essentially unchanged. The bands in zymograms from three experiments were scanned, and the quantified data showing the relative change (*x*-fold) in MMP-9 protein are summarized in Figure 1B. Doxycycline at a concentration of 10 μg/mL completely abolished the stimulation of MMP-9 by 1 ng/mL TGF-β1.

The MMP-9 activity assays confirmed the zymography results. As shown in Figure 1B, MMP-9 activities in the conditioned media from TGF-β1 (0.1, 1.0, or 10 ng/mL) treated epithelial cultures were 1.97 $\pm$ 0.20, 4.09 $\pm$ 0.10, and 4.65 $\pm$ 0.25 ng/mL (mean $\pm$ SD, n = 3) respectively, significantly higher than the control group (0.82 $\pm$ 0.09 ng/mL). MMP-9 activity in the conditioned media was significantly increased to 4.99- and 5.67-fold (both $P < 0.05$) by TGF-β1 at concentrations of 1 and 10 ng/mL, respectively. The upregulation of MMP-9 by 1 ng/mL TGF-β1 was inhibited 54.7% by 10 μg/mL doxycycline ($P < 0.05$), but MMP-9 stimulated by the higher dose of TGF-β1 (10 ng/mL) was not inhibited by this pharmacologic dose of doxycycline.

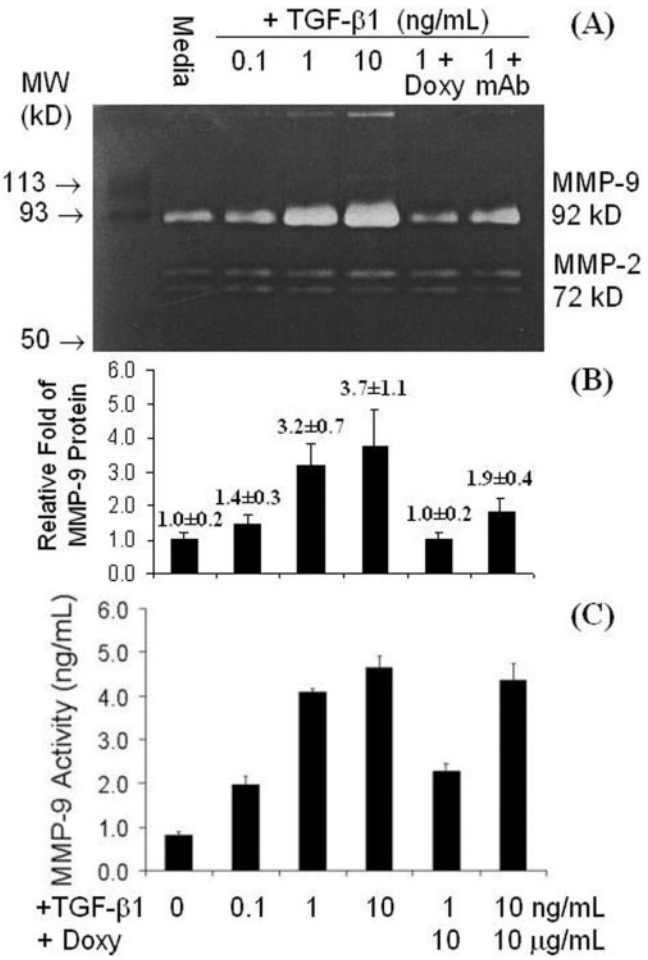


FIGURE 1. Zymography (**A**, **B**) and MMP-9 activity assay (**C**) showing MMP-9 production and activity in the conditioned media of human corneal epithelial cells treated by TGF-β1 (0.1–10 ng/mL), alone or with TGF-β1 (1 ng/mL) plus doxycycline (10 μg/mL) or a neutralizing mAb (5 μg/mL) against TGF-β1. The bands in zymograms from three experiments were scanned and quantified. Data showing relative change of MMP-9 protein (*x*-fold) are summarized in (**B**).

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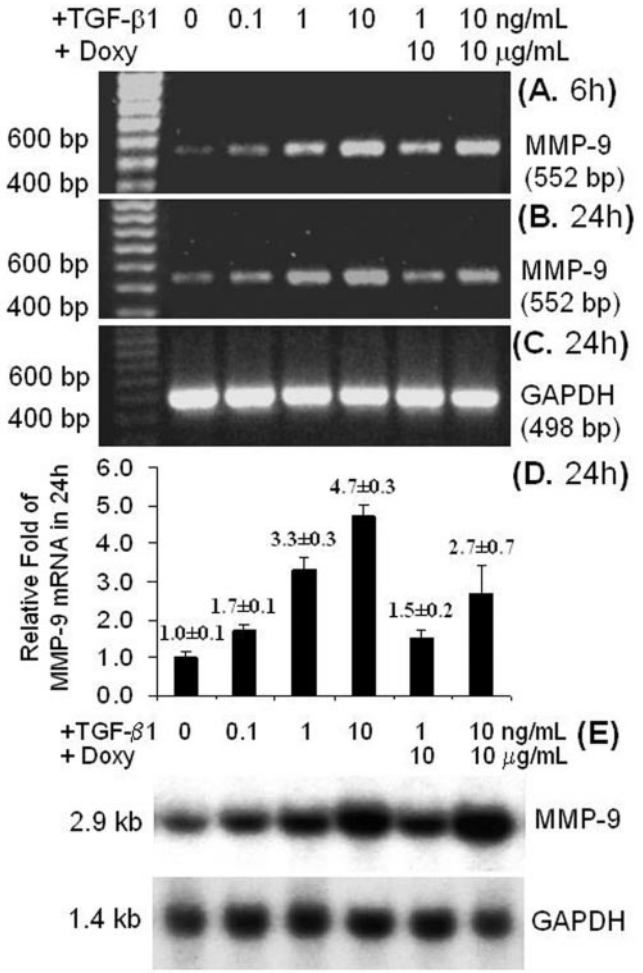


FIGURE 2. Semiquantitative RT-PCR (**A**–**D**) and Northern blot (**E**) showing the regulation of MMP-9 mRNA in human corneal epithelial cells by TGF-β1 and doxycycline at 6 (**A**, **E**) and 24 hours (**B**–**D**). The scanned and quantified data of RT-PCR profiles treated for 24 hours from three experiments (**B**, **C**) are summarized in (**D**), showing the relative change (*x*-fold) of MMP-9 mRNA after normalization by GAPDH mRNA.

Effects of TGF-β1 and Doxycycline on MMP-9 mRNA Expression

Semiquantitative RT-PCR analysis revealed that MMP-9 mRNA was expressed by normal corneal epithelial cells. The MMP-9 transcripts were upregulated in a concentration-dependent manner after 6 (Fig. 2A) and 24 hours (Fig. 2B) of treatment with increasing concentrations (0.1, 1.0, 10 ng/mL) of TGF-β1 when compared with constantly expressed levels of GAPDH mRNA (Fig. 2C), which served as an internal control. The addition of doxycycline did not affect the levels of MMP-9 mRNA at 6 hours, but did inhibit MMP-9 expression at 24 hours (Fig. 2B). The scanned and quantified data of RT-PCR profiles of cultures treated for 24 hours from three experiments (Figs. 2B, 2C) are summarized in Figure 2D, which shows the relative *x*-fold change of MMP-9 mRNA after normalization by GAPDH mRNA. The results show that 1 ng/mL TGF-β1 increased MMP-9 mRNA by 3.3-fold, whereas doxycycline decreased this stimulation by 78.3% to 1.5-fold. Northern hybridization disclosed that a single 2.9 kb band of MMP-9 transcript was expressed by cultured corneal epithelial cells (Fig. 2E). The size of this transcript is consistent with previous reports.[30] The expression of MMP-9 mRNA was stimulated in a concentration-dependent manner by treatment with different doses (0.1, 1.0, and 10 ng/mL) of TGF-β1. In contrast, the 1.4-kb band of GAPDH transcripts was unaltered by exposure to TGF-β1. The addition of doxycycline did not affect the levels of MMP-9 expression at 6 hours (Fig. 2E), which agreed with RT-PCR results.

Effects of TGF-β1 and Doxycycline on Activation of Smad Pathway

Phosphorylated Smad2 protein (60 kDa) was detected in corneal epithelial cells by Western blot using a phospho-specific polyclonal antibody. As shown in Figure 3A, 10 ng/mL TGF-β1 activated phosphorylation of Smad2 by 5 minutes after exposure and activation peaked at 30 minutes. TGF-β1 concentration dependently induced Smad2 phosphorylation, which was detected after treatment with a low concentration of TGF-β1 (0.1 ng/mL) and peaked at 10 ng/mL. Doxycycline at 10 μg/mL markedly inhibited the activation of Smad2 induced by 1 ng/mL TGF-β1 (Fig. 3B).

Effects of TGF-β1 and Doxycycline on Activation of MAPK Pathways

The activation of MAPK signaling pathways in corneal epithelial cells was detected by Western blot using phospho-specific antibodies. As shown in Figure 4A, 10 ng/mL TGF-β stimulated phosphorylation of JNK1 (46 kDa) and JNK2 (54 kDa) as early as 5 minutes, peaking at 15 minutes. TGF-β1 at 0.1, 1, and 10 ng/mL concentration dependently induced p-JNK1/2 when compared with constant levels of total JNK1/2. SP600125, a specific inhibitor of the JNK pathway, markedly inhibited the activation of JNK1/2 induced by 1 ng/mL TGF-β1, but did not decrease levels of total JNK1/2 (Fig. 4B). Doxycycline showed an inhibitory effect on the TGF-β1–induced activation of JNK1/2, especially at a 10-μg/mL concentration, which was similar to the inhibitory effect produced by SP600125 (Figs. 4B, 4C). Activation of 46-kDa p-JNK1 appeared more pronounced than 54-kDa p-JNK2, suggesting that p-JNK1 may be the major isoform of JNK that is activated by TGF-β1.

As shown in Figure 5A, Western blot revealed that 10 ng/mL TGF-β1 activated phosphorylation of ERK1/2 as early as 15 minutes, peaking at 60 minutes in the time course tested. TGF-β1, at 0.1, 1, and 10 ng/mL, concentration dependently

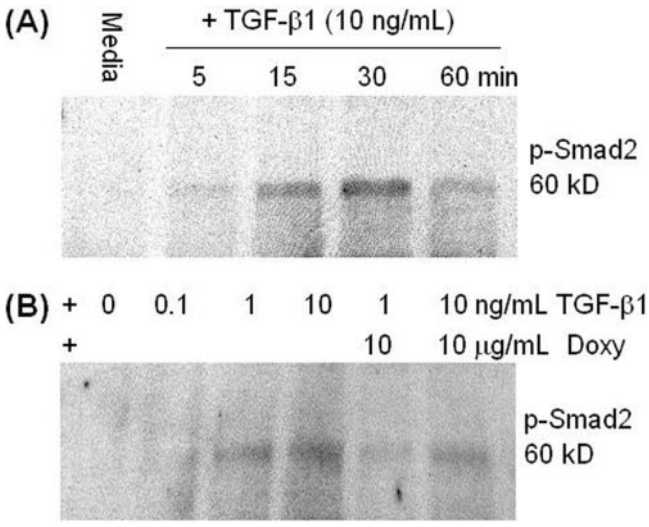


FIGURE 3. Western blot showing the time course (**A**) and dose–response (**B**) of phosphorylated Smad2 (p-Smad2) activated by TGF-β1 and inhibited by doxycycline (**B**) in human corneal epithelial cells.

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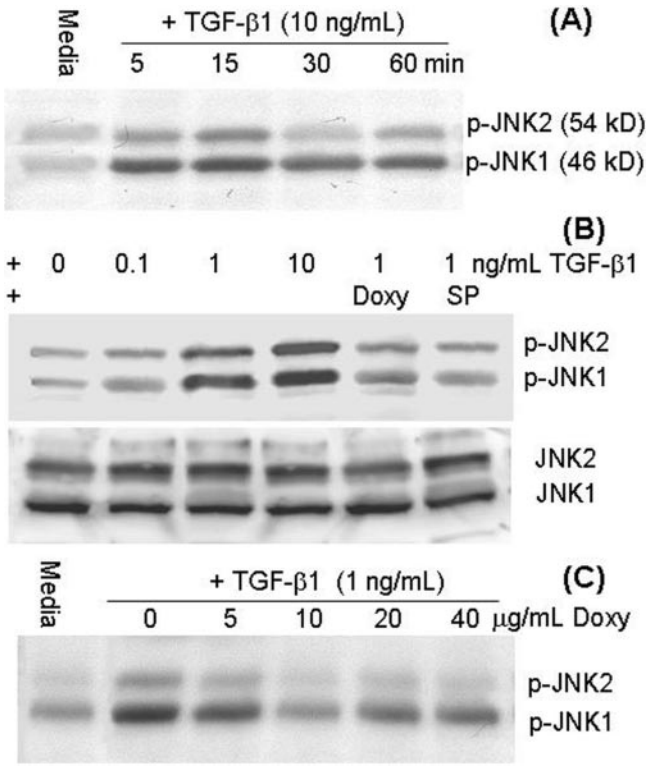


FIGURE 4. Western blot showing the time course (**A**) and dose–response (**B**) of p-JNK1 and p-JNK2 activated by TGF-β1 and inhibited by (**B**) 10 and (**C**) 5 to 40 μg/mL doxycycline (Doxy) and 30 μM SP600125 (SP) in human corneal epithelial cells.

induced p-ERK1/2 when compared with relatively constant levels of total ERK1/2. PD98059, a specific inhibitor of the ERK pathway, markedly inhibited the activation of ERK1/2 induced by 1 ng/mL TGF-β1, but did not inhibit total ERK1/2 (Fig. 5B). Doxycycline at a 5- to 40-μg/mL concentration dependently inhibited the TGFβ1-induced activation of ERK1/2 (Fig. 5C). The inhibitory effect of doxycycline on p-ERK1/2 was similar to PD98059 (Fig. 5B) All figures show that the 42-kDa band of p-ERK2 was stronger than 44-kDa p-ERK1, which may suggest that p-ERK2 was the major isoform of ERK is activated by TGF-β1.

TGF-β1 also induced activation of p38 MAPK in corneal epithelial cells. Western blot showed that 10 ng/mL TGF-β1 activated phosphorylation of p38 as early as 5 minutes, and the reaction peaked at 60 minutes in the time course tested (Fig. 6A). TGF-β1 concentration dependently induced p-p38 at a low concentration of 0.1 ng/mL, with peak activation at 10 ng/mL. In contrast, levels of total p38 remained constant. SB202190, a specific inhibitor of p38 MAPK pathway, markedly inhibited p38 phosphorylation induced by 1 ng/mL TGF-β1, but did not affect levels of total p38. Doxycycline at, 5 to 40 μg/mL, concentration dependently inhibited the TGFβ1–induced activation of p-38 (Fig. 6C). The inhibitory effect of doxycycline on p-p38 was similar to SB202190 (Fig. 6B).

These MAPK pathway inhibitors not only blocked the phosphorylation of the JNKs, ERKs, or p38, then also markedly inhibited MMP-9 production by corneal epithelial cells. SP600125, PD98059, and SB202190 almost abolished the production and activity of MMP-9 stimulated by TGF-β1 (1 ng/mL) in the cultures treated for 24 (Fig. 7A) and 48 (Fig. 7B) hours. SB202190 showed stronger inhibition of TGF-β1–induced MMP-9 than did SP600125 and PD98059, perhaps because SB202190 at 20 μM inhibits both the p38 and JNK pathways.[31,32] Doxycycline, at 5 to 40 μg/mL, concentration dependently inhibited the TGF-β1–ed production of MMP-9 (Fig. 7C). The inhibitory effect of doxycycline at 10 μg/mL on TGF-β1–stimulated MMP-9 production was similar to that of SP600125 and PD98059, whereas it was slightly weaker than that of SB202190 at 24 and 48 hours.

DISCUSSION

Stimulation of MMP-9 Expression, Production and Activity by TGF-β1

In mammals there are three isoforms of TGF-β (TGF-β1, -β2, and -β3), which are functionally similar and interchangeable under many conditions, although they may possess unique functions.[33–35] All three TGF-β isoforms are expressed by ocular surface fibroblasts and epithelial cells.[29,35–38] Among them, TGF-β1 and -β2 are abundantly present in human tears, which are secreted by lacrimal glands and are produced by the epithelial and inflammatory cells that reside on the ocular surface.[29,38,39] TGF-β1 is the predominant isoform, particularly in its latent inactive form, that may be activated by proteases during inflammation and wound healing.[40–43] In this study, we used TGF-β1 to investigate its effects on regulation of MMP-9 production by primary cultured human corneal epithelial cells.

The role of TGF-β1 in regulating MMP-9 has not been well understood, and previous studies have reported conflicting results. TGF-β1 has been reported to reduce the production of gelatinases (MMP-2 and -9) and enhance the expression of the tissue inhibitors of MMP (TIMP-1 and -3) in human lung fibroblasts[33] and myometrial smooth muscle cells.[44] TGF-β1 has also been found to stimulate production of MMP-9 in keratinocytes,[14] odontoblasts,[45] human fibroblasts,[13] and oral tumor

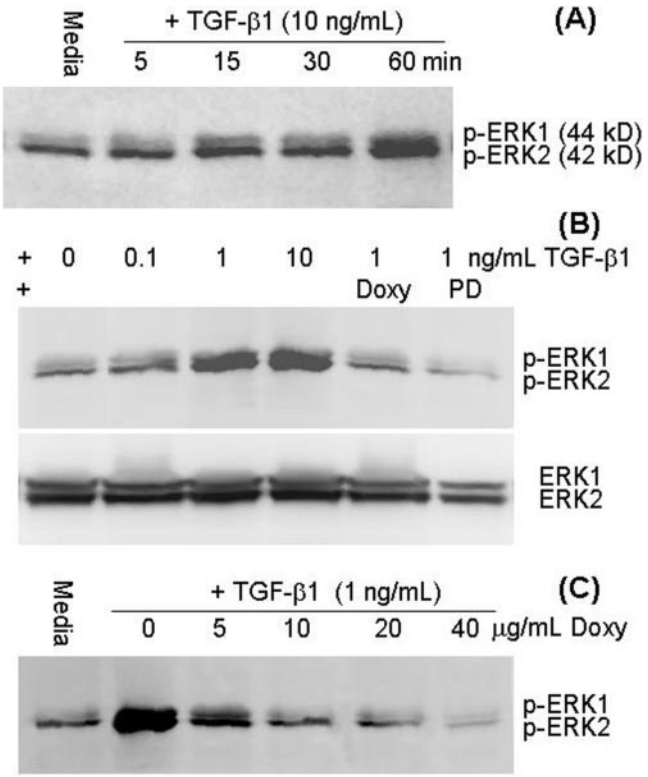


FIGURE 5. Western blot showing the time course (**A**) and dose–response (**B**) of p-ERK-1 and p-ERK-2 activated by TGF-β1 and inhibited by (**B**) 10 and (**C**) 5 to 40 μg/mL doxycycline (Doxy) and 40 μM PD98059 (PD) in human corneal epithelial cells.

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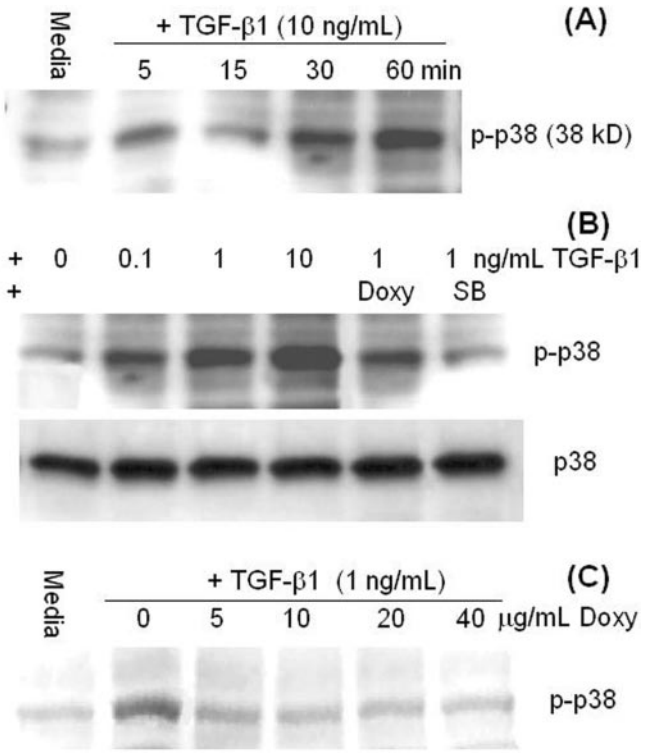


FIGURE 6. Western blot showing the time course (**A**) and dose–response (**B**) of p-p38 activated by TGF-β1 and inhibited by (**B**) 10 and (**C**) 5 to 40 μg/mL doxycycline (Doxy) and 20 μM SB202190 (SB) in human corneal epithelial cells.

cells.[46] These observations suggest that the effect of TGF-β1 on MMP-9 may be species specific, tissue or cell-type specific, and temporally and spatially specific. We have observed by zymography that TGF-β1 stimulates MMP-9 production.[11] In the current study, we demonstrated that TGF-β1 concentration dependently increased protein production and mRNA expression of gelatinase MMP-9 in cultured human corneal epithelial cells (Figs. 1, 2). This TGF-β1–stimulated MMP-9 expression may mediate epithelial cell migration during wound healing and involve the pathogenesis of ocular surface diseases such as corneal ulceration and dry eye.[47–49]

Activation of Smad and MAPK Pathways by TGF-β1

Ligands of the TGF-β superfamily are known to be unique, in that they signal through transmembrane receptor serine-threonine kinases, rather than through tyrosine kinases. The receptor complex couples to a signal transduction pathway involving a novel family of proteins, the Smads.[50] Receptor-activated Smads (Smad2 and -3) are directly phosphorylated by activated TGF-β receptors and form heteromeric complexes with Smad4 that translocate into the nucleus and modulate the transcription of target genes.[51–54] In many TGF-β-regulated genes, Smad-binding sequences are located adjacent to AP-1-recognition sites.[55,56] Although transcriptional responses can result from direct Smad binding to DNA, more commonly, functional interaction of Smads with transcriptional cofactors and coactivators or corepressors is necessary.

MAPK cascades are also a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus.[57] The kinetics of this response convincingly argue that it is a primary effect of receptor kinase activation, and the use of dominant negative type II TGF-β receptors demonstrates that the same TGF-β receptor complex is coupling to the MAPK pathways as it is to the Smad pathway.[17] It is likely that there are transcriptional responses that are uniquely Smad dependent, uniquely MAPK dependent, or interdependent on the interaction between the two. The Smad pathway is a nexus for cross talk with other signal transduction pathways and for modulation by many different interacting proteins. TGF-β receptors activate Smad-independent pathways that not only regulate Smad signaling, but also allow Smad-independent TGF-β responses.[58] Different biological responses to TGF-β1 depend to various degrees on activation of either or both of these two pathways.

MMP-9 has been observed to be regulated by TGF-β,[11,13,14] and protein kinases belonging to the MAPK family, ERK, JNK, and p38 pathways are found to involve the regulation of MMP-9 production.[59–61] The regulatory mechanisms responsible for the expression of MMP-9 by TGF-β1, however, are still poorly understood. Only a few reports have shown that TGF-β1 stimulates MMP-9 production through the Ras-ERK1/2 MAPK pathway in transformed keratinocytes.[62] In this study, TGF-β1 concentration dependently activated the phosphorylation of 60-kDa Smad2 as early as 5 minutes, with peak activity at 30 minutes. This finding is supported by a recent study showing that TGF-β–induced MMP-13 production is triggered by Smad2 protein in human osteoarthritic chondrocytes.[63] In this study, we also showed that TGF-β1 concentration dependently activates JNK1/2, ERK1/2, and p38 MAPK pathways (Fig. 4–6). The requirement for activation of these MAPK pathways in the TGF-β1–induced MMP-9 production was supported by experiments using MAPK specific inhibitors, SP600125, PD98059, and SB202190, which blocked activation of JNK/SAPK, ERK,

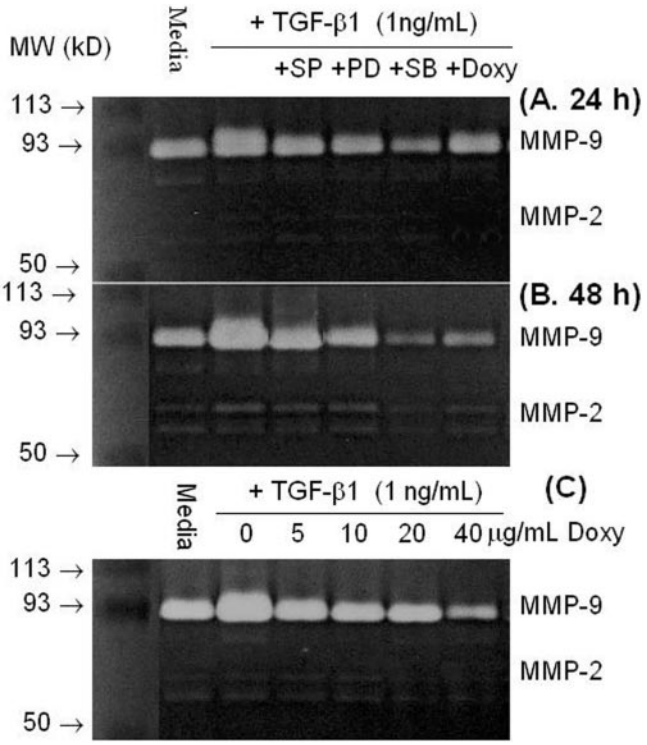


FIGURE 7. Representative zymograms showing MMP-9 production in the conditioned media of human corneal epithelial cells treated with TGF-β1 (1 ng/mL), with or without 30 μM SP600125 (SP), 40 μM PD98059 (PD), 20 μM SB202190 (SB), or 10 μg/mL doxycycline (Doxy) for (**A**) 24 or (**B**) 48 hours or 5 to 40 μg/mL doxycycline for (**C**) 24 hours.

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and p38, respectively, and also inhibited TGF-β1–induced MMP-9 production (Fig. 7). These findings demonstrate that the Smad2, JNK, ERK, and p38 pathways are involved in MMP-9 production in human corneal epithelial cells.

TGF-β1–induced activation of the ERK and JNK pathways has been found to regulate Smad phosphorylation, which facilitates both its activation by the TGF-β receptor complex and its nuclear accumulation.[64,65] Activation of MAPK pathways by TGF-β may also affect transcription responses through direct effects on Smad-interacting transcription factors allowing convergence of TGF-β–induced Smad and MAPK pathways. The dual ability of TGF-β to activate Smads and MAPK signaling has a potential role in TGF-β–induced epithelial-to-mesenchymal transdifferentiation, which depends in part on the ERK and/or p38 MAPK pathways.[16,66,67] Although this convergence often results in synergy, these pathways may also counteract each other. In this study, the relationship between the TGF-β–induced Smad and MAPK pathways was not established, and it needs further studies in the future.

Inhibition of TGF-β1–Induced MMP-9 by Doxycycline through Smad and MAPK Pathways

Doxycycline, a member of the tetracycline family, is well recognized for its therapeutic efficacy in treating ocular surface diseases, such as rosacea and sterile corneal ulceration.[19,20] Doxycycline has also been reported to inhibit the activity of MMPs, including MMP-9.[22,24,26] We have experienced clinical success in treating patients with rosacea-associated corneal epithelial erosions with doxycycline.[68] We also reported that doxycycline at a nontoxic dose, markedly decreases the production and activity of MMP-9 stimulated by IL-1β and TNF-α in cultured human corneal epithelial cells,[11,26] but the effects and mechanism of doxycycline on TGF-β–stimulated MMP-9 have not been not elucidated.

In this study, we provide evidence that doxycycline, at pharmacologically achievable nontoxic doses, concentration dependently inhibits MMP-9 protein production and mRNA expression stimulated by TGF-β1 at a concentration of 1 ng/mL, which is in the range of in vivo physiological and pathologic levels, in cultured corneal epithelial cells, as shown by zymography, activity assay, semiquantitative RT-PCR, and Northern hybridization (Fig. 1, 2). These results are supported by previous reports that doxycycline inhibits the activity of 92-kDa gelatinases in bone-metastasizing cells,[69] human skin keratinocytes,[22] and corneal epithelial cells.[11,26] We further demonstrated that doxycycline inhibits TGF-β1–induced activation of Smad2, JNK1/2, ERK1/2, and p38 MAPKs and that these inhibitory effects are similar or equal to those of SP600125, PD98059, or SB202190, the inhibitors of the JNK1/2, ERK1/2, and p38 MAPK pathways, respectively (Figs. 4–6). Therefore, doxycycline inhibits TGF-β1–induced MMP-9 production and activity, perhaps through the Smad and the three MAPK pathways.

In conclusion, TGF-β1 concentration dependently increases the expression, production, and activity of MMP-9 through activation of both Smad and MAPK signaling pathways in human corneal epithelial cells. Doxycycline dose dependently inhibits this TGF-β1–stimulated MMP-9 by blocking the activation of Smad, as well as the JNK, ERK, and p38 MAPK pathways. These findings may explain the reported efficacy of doxycycline in treating sterile corneal ulceration and other ocular surface diseases in which TGF-β1 may play a role in pathogenesis.

Acknowledgments

The authors thank the Lions Eye Bank of Texas for kindly providing human corneoscleral tissues.

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Clin Exp Immunol 1995; **102**:635–641

The influence of tetracyclines on T cell activation

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(Accepted for publication 3 August 1995)

SUMMARY

Minocycline has been shown to have an anti-inflammatory effect in patients with rheumatoid arthritis (RA). Since there is evidence that RA is a T cell-mediated disease, we investigated the effect of minocycline on human T cell clones derived from the synovium of an RA patient. The T cells, when activated via the T cell receptor (TCR)/CD3 complex, were suppressed functionally by minocycline, resulting in a dose-dependent inhibition of T cell proliferation and reduction in production of IL-2, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). Besides an inhibition of IL-2 production, minocycline exerted its effect on T cell proliferation by induction of a decreased IL-2 responsiveness. We showed that the chelating capacity of minocycline plays a crucial role in the inhibitory effect on T cell function, since the inhibitory effect on T cell proliferation could be annulled by addition of exogenous Ca^{2+}. However, minocycline did not markedly influence the typical TCR/CD3-induced intracellular Ca^{2+} mobilization. Taken together, the results clearly indicate that minocycline has immunomodulating effects on human T cells.

Keywords tetracyclines minocycline T cell function immunosuppression

INTRODUCTION

Tetracyclines are reported to have an ameliorative effect in arthritis in animals. Minocycline, a tetracycline derivative, decreased the incidence and severity in the collagen and adjuvant models of chronic arthritis in rats [1]. Additionally CMT, a chemically modified tetracycline without antibacterial activity, in combination with a non-steroidal anti-inflammatory drug, prevented bone loss in rats with adjuvant arthritis [2].

Rheumatoid arthritis (RA) is a chronic joint disease in man. In the pathogenesis of RA, T cells are held responsible for the initiation and perpetuation of ongoing synovial inflammation (for review, see [2]), which may lead to cartilage and joint destruction brought about by enzymes, especially matrix metalloproteinases.

Two open clinical studies have suggested a beneficial effect of minocycline in patients with RA [4,5]. In a double-blind placebo-controlled trial minocycline suppressed the laboratory parameters of disease activity, especially the acute-phase reactants, although the effects on clinical signs and symptoms were less pronounced [6].

Tetracyclines are predominantly known for their antimicrobial features. However, two other properties of minocycline should be considered to account for the observed phenomena in patients treated with minocycline: tetracyclines inhibit the activity of matrix metalloproteinases [7], and are immunomodulatory. In animals, tetracyclines have been shown to suppress the DTH, the rejection of transplants [8–11] and levels of serum immunoglobulin [9,12]. Furthermore, *in vitro* tetracyclines inhibited the proliferative response of human peripheral blood mononuclear cells (PBMC) to mitogens [13–16].

We studied the effect of minocycline on T cell activation, in particular in T cell clones derived from a patient with RA. In the present study, the T cells were activated by triggering the T cell receptor (TCR)/CD3 complex, which results in the generation of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Subsequently, IP_3 induces a rapid rise of intracellular free calcium ($[Ca^{2+}]_i$), whereas DAG activates protein kinase C, which together lead to the synthesis of IL-2, an autocrine growth factor of T cell proliferation. The effect of minocycline on several aspects of T cell activation were studied in more detail.

MATERIALS AND METHODS

Reagents

Minocycline HCl, kindly provided by D. G. Lederle (Etten-Leur, The Netherlands), and doxycycline HCl, kindly provided by Pfizer (Capelle a/d IJssel, The Netherlands) were obtained in

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powder form. Minocycline HCl was dissolved in culture medium (Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml; Boehringer Mannheim, Mannheim, Germany), and fetal calf serum (FCS, 10%; GIBCO)) at a concentration of 100 μg/ml. Doxycycline HCl was dissolved in 0·02 N HCl at a concentration of 1 mg/ml and further diluted in culture medium. The antibiotic solutions were freshly prepared on the day of use and diluted to the appropriate concentrations. The concentrations used in the experiments ranged from 0·8 to 50 μg/ml. Anti-CD3 MoAbs (OKT3) were obtained from Cilag (Herenthals, Belgium). Recombinant (r) IL-2 was obtained from Cetus (Gouda, The Netherlands). Indo-1/AM was obtained from Molecular Probes (Junction City, OR).

Cell cultures

T cell clones (two $CD4^+$ (N16, N18) and one $CD8^+$ (N17)) were derived from the synovium of a patient with rheumatoid factor-positive classical RA. The cloning procedure has been described in detail [17]. In short, fragments of synovial tissue were placed in 24-well tissue culture plates (Costar 3524; Cambridge, MA) in culture medium and 5% v/v T cell growth factor (TCGF). After approximately 1 week, growing cells were separated from the tissue fragments and cloned by limiting dilution in a feeder cell mixture consisting of irradiated (30 Gy) allogeneic PBMC (10^6/ml), OKT3 ascites (10^{-5} dilution), and 5% v/v TCGF. Clones were expanded and restimulated every 7 days with a feeder cell mixture. In between the clones were maintained in culture in the presence of 5% v/v TCGF.

Proliferation experiments

Overnight 96-well tissue culture plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated at room temperature with anti-CD3 MoAb (0·1 μg/ml; 0·1 ml/well) diluted in PBS. Before use the plates were washed three times with PBS. Cloned T cells were washed free of TCGF and plated at a concentration of 2×10^5 cells/well. The cells were incubated in the presence or absence of the drug for 48 h at 37°C, 5% CO_2. Twenty-four hours after initiation of the experiment, ^{3}H-thymidine (20 μCi/ml; 0·05 ml/well) was added. The cells were harvested with a Skatron AS semiautomatic cell harvester on filter paper discs. The rate of DNA synthesis was measured by liquid scintillation counting of the air-dried discs in a 1217 Rackbeta liquid scintillation counter. Experiments were performed in triplicate.

Cytokine production experiments

Washed cloned T cells (1×10^6 or 2×10^6 cells/well) were stimulated with plastic-immobilized anti-CD3 MoAb (0·1 μg/ml; 0·5 ml/well) in 24-well tissue culture plates with or without the drug for 24 h at 37°C, 5% CO_2. Cell-free culture supernatants were stored at −20°C.

Interferon-gamma assay

For the determination of interferon-gamma (IFN-γ) an ELISA [18] (kindly provided by Dr P. Van der Meide, Rijswijk, The Netherlands) was used. Briefly, microtitre plates were coated with anti-IFN-γ MoAb (MD2) for 3 h at 37°C. The test samples and rIFN-γ standards were incubated overnight at room temperature. The next day biotinylated anti-IFN-γ MoAb (MD1) was added, followed by the addition of streptavidin conjugated to horseradish peroxidase (HRP; Zymed, Sanbio, Uden, The Netherlands). For developing the assay, orthophenylenediamine (OPD; Sigma, St Louis, MO) in phosphate buffer pH 5·6, activated with H_2O_2, was used. The reaction was stopped with 10% H_2SO_4. All reagents and samples were diluted in PBS with 0·05% Tween (PBS–T) and 1% new born calf serum (NBCS) and between the incubation steps plates were washed three times with PBS–T. Optical densities (OD) were read in a Titertek Multiscan Plus at 492 nm. The sensitivity was 0·2 ng/ml.

Tumour necrosis factor-alpha assay

For the determination of tumour necrosis factor-alpha (TNF-α) an ELISA [19] (kindly provided by Dr W. Buurman, Maastricht, The Netherlands) was used. Briefly, immuno-Maxisorb plates were coated overnight at 4°C with 0·125 μg/well of the anti-human TNF-α MoAb 61E71 in PBS. Non-specific binding was prevented by incubation of the plates for 1 h at room temperature with bovine serum albumin (BSA; Organon Teknika, Oss, The Netherlands) 1% (w/v) in PBS. The test samples and rTNF-α standards were incubated for 2·5 h at room temperature. Subsequently, the plates were incubated with a rabbit polyclonal anti-human TNF-α immunoglobulin, followed by a peroxidase-conjugated goat anti-rabbit IgG. Next, the plates were washed three times with distilled water containing 0·1% Tween, and developed with ABTS activated with H_2O_2. The reaction was stopped with 2% oxalate acid. Reagents and samples were diluted in PBS–0·1% BSA (w/v). Finally, OD were read at 405 nm, using a Titertek Multiscan Plus. The sensitivity was 10 pg/ml.

IL-2 assay

IL-2 was determined by an ELISA (Boehringer Mannheim), according to the manufacturer's description. The sensitivity of the ELISA was 62·5 pg/ml.

Determination of IL-2 receptor

For determination of IL-2R (CD25) expression flow cytometric analysis was used. Per sample, 2×10^5 cells were incubated with anti-Tac MoAb (the hybridoma producing anti-Tac was obtained from the American Type Culture Collection (ATCC, Rockville, MD)) for at least 30 min on ice. After two washes with ice-cold PBS containing 1% (w/v) BSA, cells were incubated with FITC-conjugated goat anti-mouse IgG (Nordic Immunological Labs, Tilburg, The Netherlands) diluted 1:40 in PBS–1% BSA. Cells were washed twice and analysed on a FACStar (Becton Dickinson, Mountain View, CA). The experiment was performed in duplicate on two separate occasions.

Measurement of intracellular free calcium

$[Ca^{2+}]_i$ was measured as described before [20]. In short, prewarmed Jurkat cells (5 min at 37°C, 10×10^6/ml) were resuspended in HEPES medium (NaCl (133 mM), KCl (6 mM), $MgSO_4$ (1 mM), $CaCl_2$ (1 mM), potassium phosphate (1 mM), glucose (5·5 mM), HEPES (20 mM), and human albumin (0·5% w/v), pH 7·4) and loaded with 1 μM Indo-1/AM during 45 min at 37°C. Subsequently, the cells were washed, resuspended at 10×10^6 cells/ml in the HEPES medium and kept at room temperature. Fluorescence measurements were performed at

FOI 26-3132 - Document 5

37°C in stirred suspensions after dilution of the cells to 1×10^6/ml. Calibration of Indo-1 fluorescence as a function of $[Ca^{2+}]_i$ was determined as follows: to saturate all trapped Indo-1 with Ca^{2+}, 5 μM digitonin was added to the cell suspension. Subsequently, the Indo-1 signal was quenched by adding 0·5 mM Mn^{2+}. $[Ca^{2+}]_i$ was calculated as described [21], using 250 nM as the K_d for the Indo-1/Ca^{2+} complex.

Viability

Cell viability was determined by trypan blue exclusion and measurement of lactate dehydrogenase activity and the concentration of β_2-microglobulin in the supernatant. Lactate dehydrogenase activity was determined on a BM/Hitachi 747 (Boehringer Mannheim, Almere, The Netherlands) using an ultraviolet method according to the manufacturer's instructions. Levels of β_2-microglobulin were determined by IMx β_2-microglobulin assay (Abbott Labs, Santa Clara, CA), which is a microparticle enzyme immunoassay. The assay was performed according to the manufacturer's instructions. The sensitivity of the test is 5 $\mu g/l$.

Statistical analysis

The effect of the drug was expressed as percentage inhibition, which is defined as (1 − (effect in the presence of the drug/effect in the absence of the drug)) × 100%.

Non-parametric tests were used to determine statistically significant differences.

The correlation coefficient between the effects of the different doses of minocycline and doxycycline was estimated by means of the intraclass correlation coefficient. This was calculated using multivariate analysis of variance (MANOVA) with dose as a within-subjects factor.

RESULTS

Minocycline inhibits TCR/CD3-induced proliferation in $CD4^+$ and $CD8^+$ synovial T cell clones

In one $CD8^+$ (N17) and two $CD4^+$ (N16, N18) synovial T cell clones proliferation was induced with plastic-immobilized anti-CD3 MoAb in the presence or absence of minocycline. Minocycline inhibited the proliferation of each T cell clone in a dose-response manner (Fig. 1).

Cell viability, as measured with trypan blue staining, was >80% with all drug concentrations tested. Accordingly, no significant differences in lactate dehydrogenase release could be measured in the presence of minocycline at concentrations of 25 and 50 μg/ml. Moreover, the production of the constitutively expressed protein β_2-microglobulin was measured to investigate the viability of the cells. In unstimulated T cells (1×10^6 cells) the total β_2-microglobulin concentration was 85 ± 27 ng/ml (mean ± s.d.). A slight increase in β_2-microglobulin production to 111 ± 32 ng/ml was observed when T cells were stimulated via the TCR/CD3 complex for 24 h; in the presence of 25 μg/ml minocycline the production of β_2-microglobulin, being 94 ± 26 ng/ml, was always more than the amount of β_2-microglobulin produced by resting T cells.

Since minocycline affected all three clones to the same extent, we continued our experiments with one representative clone (N18).

Minocycline inhibits TCR/CD3-induced IL-2, IFN-γ and TNF-α production by a $CD4^+$ synovial T cell clone

Stimulation of the $CD4^+$ T cell clone N18 by anti-CD3 MoAb coated to tissue culture plates induced the production of IL-2, IFN-γ and TNF-α. Minocycline inhibited IL-2 production in a dose-dependent manner. Minocycline at a concentration of 25 μg/ml inhibited IL-2 production 26 ± 7·5% (mean ± s.e.m.) (Fig. 2). Minocycline also inhibited IFN-γ and TNF-α production in a dose-response manner. The maximal percentage inhibition was 23·9 ± 3·1% and 21·5 ± 5% for IFN-γ and TNF-α production, respectively, at a concentration of 25 μg/ml minocycline (Fig. 2).

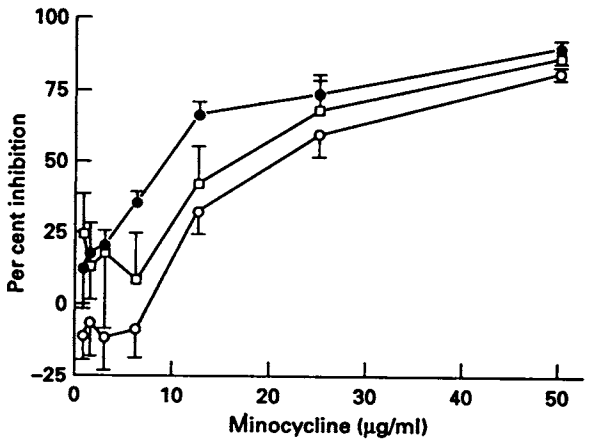


Fig. 1. The effect of minocycline on anti-CD3-induced proliferation of synovial T cell clones (○, N16; □, N17; ●, N18). The symbols reflect mean ± s.e.m. of at least three experiments. The results are presented as percentage inhibition of control in the absence of minocycline. The control ranged from 12 351 to 45 729, from 17 132 to 72 985, and from 10 118 to 64 794 ct/min for N16, N17 and N18, respectively.

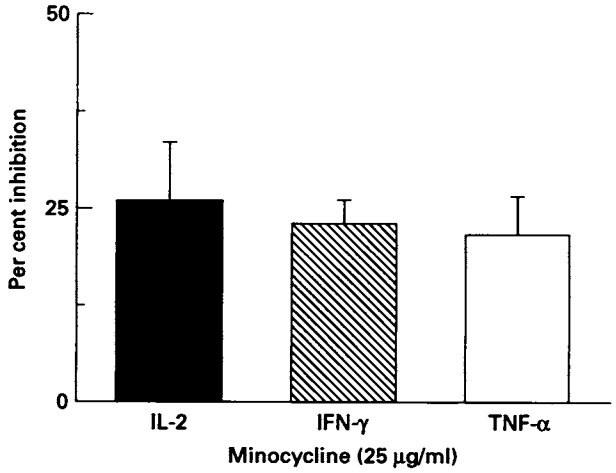


Fig. 2. The effect of minocycline on anti-CD3-induced IFN-γ and tumour necrosis factor-alpha (TNF-α) production by cloned T cells (N18). Shown are mean ± s.e.m. of three, five and six experiments for IL-2, IFN-γ and TNF-α, respectively, in the presence of 25 μg/ml minocycline. The results are described as percentage inhibition of induced production in the absence of minocycline. The production of the individual cytokines ranged from 625 to 7820 pg/ml for IL-2, from 92 to 1197 ng/ml IFN-γ, and from 7 to 40 ng/ml for TNF-α.

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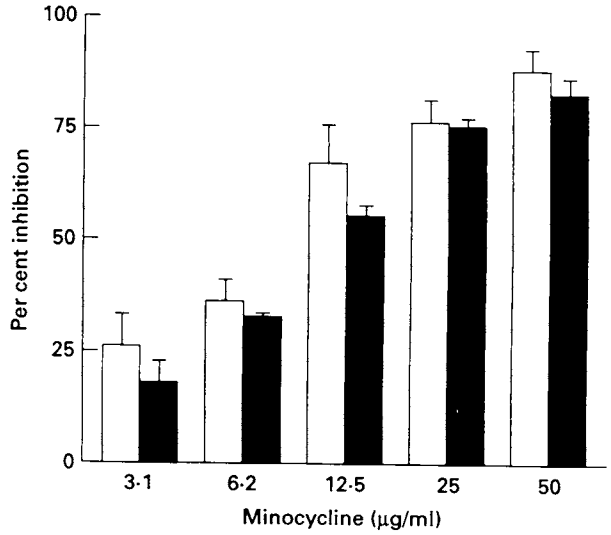


Fig. 3. Inhibition of anti-CD3-induced proliferation in cloned T cells (N18) by minocycline could not be restored by the addition of exogenous recombinant (r)IL-2. Shown are mean ± s.e.m. of three experiments. The results are described as percentage inhibition of control. The control ranged from 10 903 to 68 458 and from 16 158 to 56 641 ct min for 0 and 10 U ml IL-2, respectively. □ and ■ represent, respectively, addition of 0 and 10 U ml rIL 2.

Minocycline inhibits IL-2 responsiveness in synovial T cell clones

Next, we determined whether the inhibitory effect of minocycline on T cell proliferation could be bypassed by the addition of IL-2. The $CD4^+$ T cell clone is known to express IL-2R and to respond to IL-2 addition by proliferation. However, the addition of rIL-2 (0·1, 1 or 10 U/ml) did not bypass the inhibitory effect of minocycline on TCR CD3-induced proliferation in cloned T cells (Fig. 3). In proliferation experiments in which cloned T cells were stimulated with rIL-2 (20 U ml) in the absence or presence of minocycline, an inhibition of T cell proliferation by minocycline in a dose-dependent manner was seen (Fig. 4).

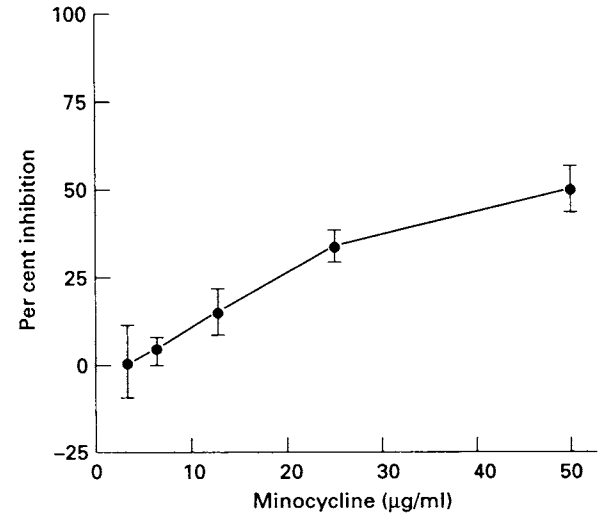


Fig. 4. The effect of minocycline on IL-2-induced proliferation in cloned T cells (N18). The symbols represent mean ± s.e.m. of three experiments. The results are described as percentage inhibition of control. The control ranged from 44 385 to 75 895 ct min.

To investigate whether inhibition of IL-2 responsiveness was caused by down-regulation of the expression of the IL-2R α chain (CD25) by minocycline, cloned synovial T cells were stimulated with anti-CD3 MoAb in the presence or absence of minocycline for 24 h and subsequently analysed for IL-2R expression using anti-Tac MoAb. In the absence of minocycline 86–92% of T cells were positive for IL-2R (mean fluorescence intensity ranging from 518 to 544) against 86–96% in the presence of 50 μg/ml minocycline (mean fluorescence intensity ranging from 437 to 542).

Doxycycline, like minocycline, inhibits TCR/CD3-induced T cell proliferation and IFN-γ production

To study whether the effect on T cell activation was a more general property of tetracyclines, another tetracycline derivative, i.e. doxycycline, was studied also. Doxycycline and minocycline have corresponding biochemical characteristics with regard to lipid solubility and chelating capacity.

Both drugs inhibited anti-CD3-induced proliferation in a concentration-dependent way and to the same extent (MANOVA, $r = 0{\cdot}65$; $P = 0{\cdot}064$) (Fig. 5a). The inhibitory capacity of doxycycline and minocycline towards TCR/CD3-induced IFN-γ production was similar (MANOVA, $r = 0{\cdot}71$; $P = 0{\cdot}001$) (Fig. 5b). Similar to minocyclin we observed no toxic effects of doxycycline on T cell functioning in the presence of the concentrations tested.

The inhibitory effect of minocycline on proliferation is partially reversible

When cloned synovial T cells were preincubated with minocycline at a concentration of 50 or 12·5 μg/ml for 24 h at 37 C, 5% CO_2, subsequently harvested, washed, counted, adjusted to cell number and finally incubated on plates coated with anti-CD3 MoAb, we also observed inhibition of T cell proliferation. However, the inhibitory effect of preincubation with 50 or 12·5 μg ml minocycline was less, namely 30% and 19%, than the inhibitory effect of direct incubation with minocycline, being 76% and 35% (Fig. 6).

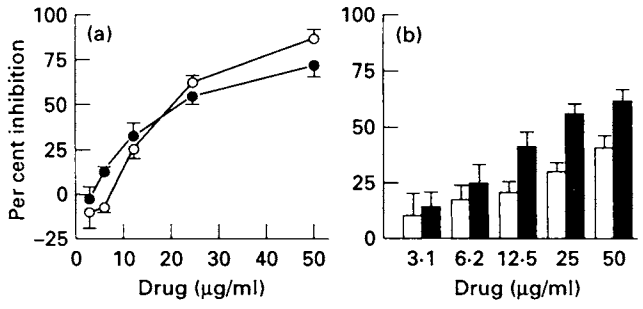


Fig. 5. The effect of doxycycline and minocycline on anti-CD3-induced proliferation (a) and IFN-γ production (b) by cloned T cells (N18). The symbols reflect the mean ± s.e.m. values of three and five separate experiments for proliferation and IFN-γ production, respectively. The results are described as percentage inhibition of control. The control values for the proliferation ranged from 17 122 to 44 431 ct min and for IFN-γ production from 220 to 1560 ng ml. ○, □, Minocycline; ●, ■, doxycycline.

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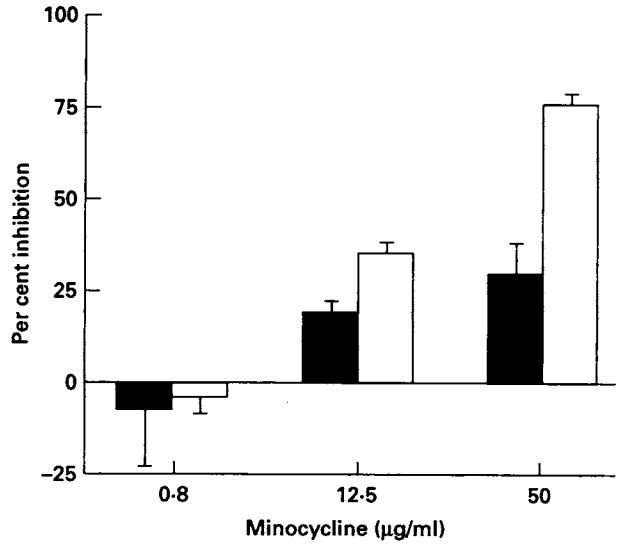


Fig. 6. The inhibitory effect of minocycline on T cell proliferation was partially reversible. Cloned T cells (N18) were preincubated with or without minocycline for 24 h at 37°C, 5% CO_2. Afterwards the cells were harvested, washed, counted, adjusted to cell number, and proliferation was induced with anti-CD3 MoAb (0·1 μg/ml). As controls, cells which had been preincubated without minocycline were incubated with minocycline. Symbols represent mean ± s.e.m. of four separate experiments. Results are described as percentage inhibition relative to proliferation in the absence of minocycline, which ranged from 12 976 to 40 040 ct/min. □, Proliferation experiments in the presence of minocycline; ■, proliferation experiments after preincubation with minocycline.

Addition of Ca^{2+} restores the inhibition of TCR/CD3-induced proliferation by minocycline

Since minocycline is a compound with chelating capacity [22], we studied whether Ca^{2+} plays a role in the inhibition of TCR/CD3-induced proliferation by minocycline.

Therefore, exogenous Ca^{2+}, ranging from 1·6 to 9·6 mM, was added to cloned synovial T cells, stimulated with plastic-immobilized anti-CD3 MoAb in the absence or presence of minocycline. The inhibitory effect of minocycline was partially abolished at low concentrations of Ca^{2+} in the culture medium and totally abolished at high concentrations of Ca^{2+} in the culture medium (Fig. 7a,b).

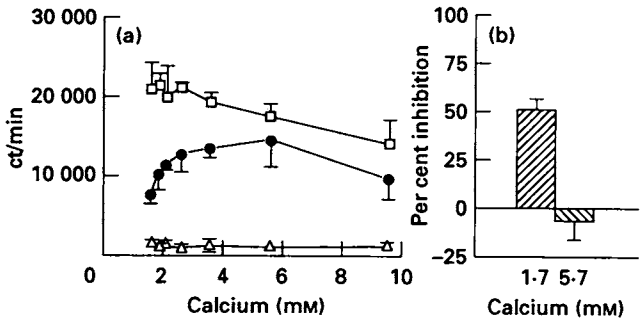


Fig. 7. Addition of Ca^{2+} restored the inhibition of anti-CD3-induced proliferation by minocycline. (a) Ca^{2+} was added in proliferation experiments of cloned T cells (N18). (Δ, Proliferation in medium without anti-CD3 stimulus; □, proliferation induced with plastic-immobilized anti-CD3 (0·1 μg/ml); ●, anti-CD3-induced proliferation in the presence of 25 μg/ml minocycline.) (b) Percentage inhibition by 25 μg/ml minocycline compared with control in the presence of a low and high concentration of Ca^{2+} in the culture medium. The results (mean ± s.e.m.) of four independent proliferation experiments are demonstrated.

Minocycline does not affect TCR/CD3-induced rises in $[Ca^{2+}]_i$ in Jurkat cells

To study whether minocycline affected $[Ca^{2+}]_i$ mobilization upon TCR/CD3 triggering, we studied Jurkat T cells, which are generally used as a model for resting T cells and particularly for studying $[Ca^{2+}]_i$ mobilization [23]. In a series of experiments (n = 10) on three separate occasions the basal $[Ca^{2+}]_i$ of 58 ± 24 nM increased after anti-CD3 stimulation to 222 ± 186 nM, followed by a plateau of 93 ± 26 nM. When minocycline (25 μg/ml) was present in the experiments (n = 9) the basal $[Ca^{2+}]_i$ was 115 ± 31 nM, increased to 222 ± 136 nM, and was followed by a plateau of 168 ± 75 nM. Minocycline did not influence the typical $[Ca^{2+}]_i$ response, but increased the basal and plateau levels of $[Ca^{2+}]_i$ ($P < 0·05$).

DISCUSSION

The present study shows that minocycline exerts immunomodulatory effects on human T cells.

Tetracyclines have apart from their immunomodulatory features, other properties that validate their usage as a treatment modality in RA. For instance, tetracyclines inhibit the enzyme activity of metalloproteinases [7], including synovial collagenases [24]. Therefore, cartilage and bone breakdown may be decreased as a result of treatment with tetracyclines. Concordantly, prophylactic treatment with oral doxycycline reduced the severity of canine osteoarthritis [25]. In addition, tetracyclines inhibit the activity of phospholipase A_2 [26], suppress the function of neutrophils [27,28] and scavenge oxygen radicals [29], which may result in an anti-inflammatory effect. Diminishing of joint destruction and inflammation may have a beneficial effect in RA.

In the literature, tetracyclines have been shown to inhibit the proliferative response of human T cells in monocyte-dependent, mitogen-stimulated assay systems [13–16]. Whether or not minocycline directly affects T cell proliferation *per se* is difficult to conclude on the basis of these studies in complex cell systems, especially since tetracyclines have been reported to influence monocyte functioning [30]. The present study investigated the effect of minocycline on T cells selectively and showed that minocycline affected both TCR/CD3 as well as IL-2-induced proliferation, which was due to a negative effect on IL-2 production and on IL-2 responsiveness. Besides an inhibitory effect on IL-2 production, minocycline also negatively influenced the production of the other early induced cytokine gene products IFN-γ and TNF-α [31]. Similar results were obtained with peripheral blood T cells, whereas minocycline exerted stimulatory effects on cytokine production by monocytes (Kloppenburg and Verweij, unpublised observations). These findings indicate that minocycline affects cell function in a cell-specific manner.

Since T cell-targeted therapy, including thoracic duct drainage, total lymph node irradiation, lymphocytapheresis and cyclosporin A, exerts beneficial effects in RA, compounds with immunosuppressive properties are generally considered to

FOI 26-3132 - Document 5

be interesting candidates for treatment in RA. However, despite the effect of minocycline on T cell function *in vitro*, the effect of minocycline treatment in patients with RA was not very strong [5]. That not all T cell-targeted therapies lead to a beneficial effect in RA has also been observed in recent work of our group on the treatment of early RA with CD4 MoAbs in a placebo-controlled trial [32,33]. Despite a decline in the number of circulating $CD4^+$ cells and in histological scores for CD3, CD4, CD45RO, CD45RA, and CD38, no clinical improvement has been seen.

Tetracyclines are chelators of divalent cations [23], which may account for several features of tetracyclines. Addition of divalent cations (Ca^{2+}) *in vitro* overcomes the inhibition of metalloproteinase activity [7] and the suppression of neutrophil functions [29,34] by tetracyclines. In the present study we show that the inhibitory effect of minocycline on T cell proliferation could be overcome by addition of Ca^{2+}. The inhibitory effect was found to be higher in the continued presence of minocycline than after preincubation with the drug. Together, these findings suggest that the chelating activity of minocycline is important for the inhibition of T cell proliferation. The chelating effect of tetracyclines may possibly interfere with T cell proliferation in two ways. First, tetracylines have been shown to bind to DNA by calcium bridges [35], which may account for the inhibition of T cell proliferation. Moreover, in the association curve of DNA and tetracyclines an optimum is observed for the amount of calcium that binds to DNA [35]. Possibly, the addition of extra calcium changed the composition of the calcium–tetracycline complexes in such a way that these complexes bound less efficiently to DNA and therefore did not induce inhibition of T cell proliferation. Second, minocycline may interfere with early signal transduction, since minocycline has been shown to negatively influence the early activated genes upon TCR/CD3 stimulation (Fig. 2). Triggering of the TCR/CD3 complex results in an increased $[Ca^{2+}]_i$ which is obligatory for T cell proliferation [23,36]. The chelating capacity of minocycline may prevent the increase in $[Ca^{2+}]_i$. However, our results have demonstrated no inhibitory effect of minocycline on $[Ca^{2+}]_i$ mobilization following anti-CD3-induced T cell activation, but in contrast have shown an increase in the value of basal $[Ca^{2+}]_i$. On the other hand, these results agree with an *in vitro* study in osteoclasts showing a transient elevation of $[Ca^{2+}]_i$ after application of minocycline [37]. Accordingly, T cells from rats, treated orally with minocycline, have shown an increase in $[Ca^{2+}]_i$ upon concanavalin A stimulation *in vitro* compared with T cells from untreated rats [38].

In the present study minocycline has been shown to affect the IL-2 responsiveness of T cell clones. In the literature minocycline has been reported to inhibit the mitogen-induced up-regulation of IL-2R in peripheral T cells [16]. Synovial T cell clones, used in the present study, express IL-2R constitutively, and minocycline did not down-regulate the expression of these receptors. Thus, a diminished IL-2R expression can not explain the observed inhibition of proliferation.

In general, prediction of *in vivo* potency of drugs on the basis of *in vitro* results is limited. Serum concentrations of minocycline after a normal oral dose regimen (initial oral dose of 200 mg followed by 100 mg every 12 h thereafter) ranged from 2·3 to 3·5 μg/ml [39]. T cell function *in vitro* was found to be inhibited at a concentration of 12·5 μg/ml minocycline or higher. Since minocycline has a high degree of lipid solubility, this compound is widely distributed in the body. This results in tissue concentrations that exceed serum concentrations [40]. Moreover, lipid-soluble tetracyclines have been reported to accumulate in leucocytes [9,41,42]. In agreement with the latter are the *in vitro* studies that demonstrated that lipid-soluble tetracyclines are more potent inhibitors of mitogen-induced proliferation than non-lipid-soluble ones [13–16].

ACKNOWLEDGMENTS

We thank Mrs H. H. de Rooy and Mrs M. J. A. Verdonk, and A. T. J. Tool (Laboratory of Experimental and Clinical Immunology, University of Amsterdam, The Netherlands), for excellent technical assistance. We also thank Dr A. H. Zwinderman (Department of Medical Statistics, University Hospital Leiden) for supporting the statistical analyses.

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FOI 26-3132 - Document 5
REVIEW
published: 17 November 2020
doi: 10.3389/fmolb.2020.606393

Combating the Pandemic COVID-19: Clinical Trials, Therapies and Perspectives

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Edited by:
Balakumar Chandrasekaran, Philadelphia University, Jordan

Reviewed by:
Gaurav Kumar Jain, Delhi Institute of Pharmaceutical Sciences and Research, University of Delhi, India
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Specialty section:
This article was submitted to Molecular Diagnostics and Therapeutics, a section of the journal Frontiers in Molecular Biosciences

Received: 14 September 2020
Accepted: 19 October 2020
Published: 17 November 2020

Citation:
Kotta S, Aldawsari HM, Badr-Eldin SM, Alhakamy NA, Md S, Nair AB and Deb PK (2020) Combating the Pandemic COVID-19: Clinical Trials, Therapies and Perspectives. Front. Mol. Biosci. 7:606393. doi: 10.3389/fmolb.2020.606393

The coronavirus disease-19 (COVID-19) is caused due to the infection by a unique single stranded enveloped RNA virus, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The COVID-19 has claimed many lives around the globe, and a promising solution to end this pandemic is still awaited. Till date neither an exact antiviral drug nor a vaccine is available in the market for public use to cure or control this pandemic. Repurposed drugs and supportive measures are the only available treatment options. This systematic review focuses on different treatment strategies based on various clinical studies. The review discusses all the current treatment plans and probable future strategies obtained as a result of a systematic search in PubMed and Science Direct database. All the possible options for the treatment as well as prophylaxis of COVID-19 are discussed. Apart from this, the article provides details on the clinical trials related to COVID-19, which are registered under ClinicalTrials.gov. Potential of drugs based on the previous researches on SARS-CoV, MERS-CoV, Ebola, influenza, etc. which fall under the same category of coronavirus are also emphasized. Information on cell-based and immunology-based approaches is also provided. In addition, miscellaneous therapeutic approaches and adjunctive therapies are discussed. The drug repurposing options, as evidenced from various *in vitro* and *in silico* models, are also covered including the possible future solutions to this pandemic.

Keywords: COVID-19, SARS-CoV-2, vaccine, convalescent plasma therapy, drug repurposing

INTRODUCTION

Coronavirus disease-19 (COVID-19) is a rapidly transmitted respiratory disease that has recently attracted the worldwide public health attention since its declaration as a pandemic by the World Health Organization (WHO) on March 11, 2020 (WHO, 2020). Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the novel coronavirus responsible for COVID-19, primarily attacks the human respiratory system. Several upsurges of coronaviruses have previously occurred, like the severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS); both syndromes were considered as significant public health warnings (De Wit et al., 2016). In December 2019, some patients in China were diagnosed with pneumonia of an undetermined underlying cause (Bogoch et al., 2020; Lu H. et al., 2020). Early reports anticipated the start of a new coronavirus

upsurge that was named by the WHO as COVID-19, on February 11, 2020. The high rate of human-to-human transmission of COVID-19 infection resulted in the necessity of patients' isolation and a great urge for social distancing (Rothan and Byrareddy, 2020; Zhao et al., 2020).

Severe acute respiratory syndrome coronavirus-2 is the most recently discovered species of the coronaviruses (CoV) that infects humans, and it is categorized as a new strain of beta CoV. The genetics of the virus revealed above 80% similarity to SARS-CoV and above 50% to the MERS-CoV that originated in bats (Lu R. et al., 2020; Nadeem et al., 2020). Studies have been conducted to find a reservoir host or intermediate transmitter of the newly emerging virus. Early reports assume that two snake species as major reservoir of COVID-19. But, no evidences have been confirmed for animals other than bats and mammals as coronavirus reservoirs (Bassetti et al., 2020; Ji et al., 2020). Human-to-human transmission has been regarded as a likely mode of COVID-19 infection based on disease spreading within families and among people who were not exposed to animals (Graham Carlos et al., 2020; Helmy et al., 2020). Human-to-human transmission has been suggested to happen by unmediated contact or via droplets that outspread by coughing or sneezing from a person infected with the virus. Accordingly, the WHO has advised for keeping a distance of 1.5–2 m between people to reduce the likelihood of infection by nose or mouth droplets. However, the possibility of virus conveyance by airborne droplets over a distance of 2 m has been suggested by recent studies (Setti et al., 2020; van Doremalen et al., 2020).

The commonly identified clinical symptoms of COVID-19 disease are fever, dry cough, shortness of breath and excessive tiredness. Minor signs comprise of headache, sputum production, diarrhea, and lymphopenia (Borah et al., 2020; Ren et al., 2020). The symptoms, as mentioned earlier, are mostly revealed after an incubation period of about five days (Li Q. et al., 2020). A chest computed tomography (CT) scan in people with COVID-19 presents the clinical features of pneumonia (Huang et al., 2020). However, there are additional abnormal manifestations, including acute respiratory distress, severe cardiac side effects, and the presence of ground glass like opacities were found in sub-pleural areas of lungs in many cases. These opacities are likely to cause systemic and localized immune reactions that result in an elevated inflammatory response. Sorrowfully, the treatment of some cases with interferon did not exhibit promising clinical effects. Instead, the pulmonary opacities showed progression with consequent worsening of the condition (Dong D. et al., 2020; Lei et al., 2020). Recently it was found that the disease exhibits a broad spectrum of clinical signs and symptoms. Various case studies reported the involvement of all the vital organs of the body including heart, lungs, GIT, liver, kidneys, and central nervous system (CNS). In severe cases multisystem involvement can be seen and might lead to worse clinical outcomes as well as increased mortality (Gavriatopoulou et al., 2020).

No exact antiviral drug or vaccine against COVID-19 has been discovered yet. The only available option, other than using symptom alleviating agents, is utilizing broad-spectrum antiviral drugs comprising protease inhibitors and nucleoside analogs in an attempt to attenuate the viral infection (Lu, 2020). Amongst the antivirals that are recently recorded as having some effects against COVID-19 are oseltamivir (anti-flu drug), lopinavir/ritonavir (anti-human immunodeficiency virus; anti-HIV), and ganciclovir (Costanzo et al., 2020). High efficacy of the broad-spectrum antiviral remdesivir, which has been utilized for controlling the Ebola virus and the antimalarial agents like chloroquine and hydroxychloroquine, are also reported for controlling COVID-19 infection (Kumar et al., 2020; Ledford, 2020). Moreover, there are so many other molecules that are under development and testing. This article aims at reviewing the present and possible therapeutic options for the management of this emerging and widely spreading pandemic.

METHOD

We systematically searched the researches, reviews, and case reports in PubMed and Science Direct database, using the keywords COVID-19 and treatment, and selected the relevant articles. Some of the cross-references were also accessed. After selecting the potential agents for the COVID-19 treatment, again a search was conducted using the drug name AND COVID. The search is refined by the term "2020." The paper includes all the possible treatment strategies as well as all clinical trials for treatment or prevention of COVID-19 which are registered in NIH ClinicalTrials.gov.

MECHANISM OF COVID-19 INFECTION AND POSSIBLE DRUG TARGETS

The spike glycoprotein on the viral envelope is a major determinant of entry of virus to host cells by binding with its cellular receptor, angiotensin converting enzyme 2 (ACE-2). The viral infectivity and fusion are due to a significant proteolytic cleavage episode, and through clathrin-dependent as well as independent endocytosis. Once entered into the cells, the viruses release RNA which will synthesize two polyproteins and structural proteins, and starts replication. Then, the formation of nucleocapsid takes place by the combination of genomic RNA and nucleocapsid protein. Finally, the vesicles with viral particles combine with plasma membrane and discharge the viruses (Li X. et al., 2020). Different therapeutic agents against COVID-19 target at one or more different stages of the replication cycle. The mechanism of replication COVID-19 inside host cell and possible drug targets are shown in **Figure 1**.

Various studies reported moderate to severe "cytokine storms" in severe patients. The "cytokine storm" which in turn leads to acute respiratory distress syndrome (ARDS), occurs due to the effects of combined action of several immunoactive molecules (Coperchini et al., 2020). This seems to be one of the most hazardous and life-threatening episodes in COVID-19. After binding to alveolar epithelial cells, the virus activates innate as well as the adaptive immune system. Following the SARS-CoV-2 infection macrophages are released as a response to inflammatory signals by type 2 cells. Cytokines are released by macrophages that in turn results in the release of more immune cells to the injury

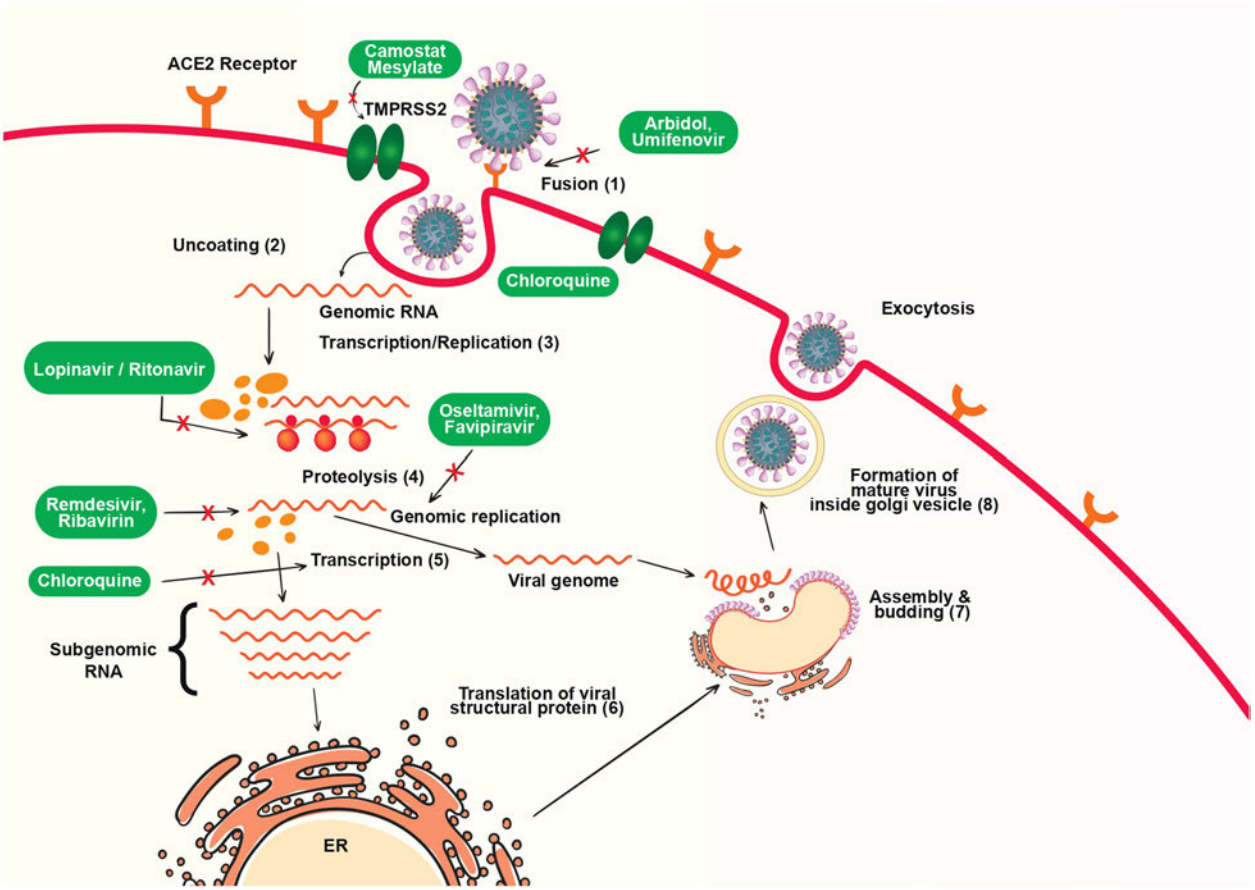


FIGURE 1 | Replication of SARS-CoV-2 and possible drug targets.

site. Cytokines cause vasodilatation also. Fluid accumulation in alveoli causes the damage of surfactant, and thus alveolar collapse which in turn affects the gas exchange. Further recruitment of neutrophils results in the release of reactive oxygen species (ROS) for destroying the infected cells. Also, the extensive release of cytokines occurs including interleukin-6 (IL-6), resulting in subsequent increase in the vascular permeability. This further leads to the entry of a large number of blood cells and fluid into the lungs, and causes dyspnea and respiratory failure (Zhang C. et al., 2020). The hyper-inflammation and cytokine storm syndrome is responsible ARDS and multi-organ failure. An illustration showing the effect of COVID-19 infection on the lungs is shown in **Figure 2**.

CURRENT TREATMENT STRATEGIES

Identifying effective therapeutic agents to fight this pandemic is urgently needed in this scenario (Li H. et al., 2020). A simplified classification of therapeutic options against COVID-19 is presented in **Figure 3**.

Drug repurposing is the only fastest approach to find potential candidates as a preventive or therapeutic measure for this new deadly pandemic (Ekins et al., 2020). Trials with ivermectin and hydroxychloroquine are examples of such an approach. At the same time, a large number of current researches are based on the testing of proven antiviral agents for related infections caused by SARS-CoV as well as MERS-CoV. The latter approach of the use of existing antiviral agents with proven efficiency against coronaviruses seems to be more promising. This argument could be justified by the fact that SARS-CoV-2 also belong to the same class of beta coronaviruses like SARS-CoV and MERS-CoV. Details of clinical trials on drug candidates against COVID-19 are presented in **Table 1**.

Antiviral Agents

Remdesivir

Remdesivir is an investigational antiviral agent and also the first drug under clinical trial in the United States as an experimental cure for COVID-19 (Holshue et al., 2020). It possesses broad spectrum of action against different RNA viruses, such as MERS-CoV. Remdesivir and chloroquine are shown to be very efficient in the control of novel coronavirus infection *in vitro* (Li Q. et al., 2020). To understand the mechanism of inhibition, in a recent study, the MERS-CoV non-structural proteins were co-expressed in insect cells as a part of the polyprotein. The research demonstrated that remdesivir acts by inhibiting the

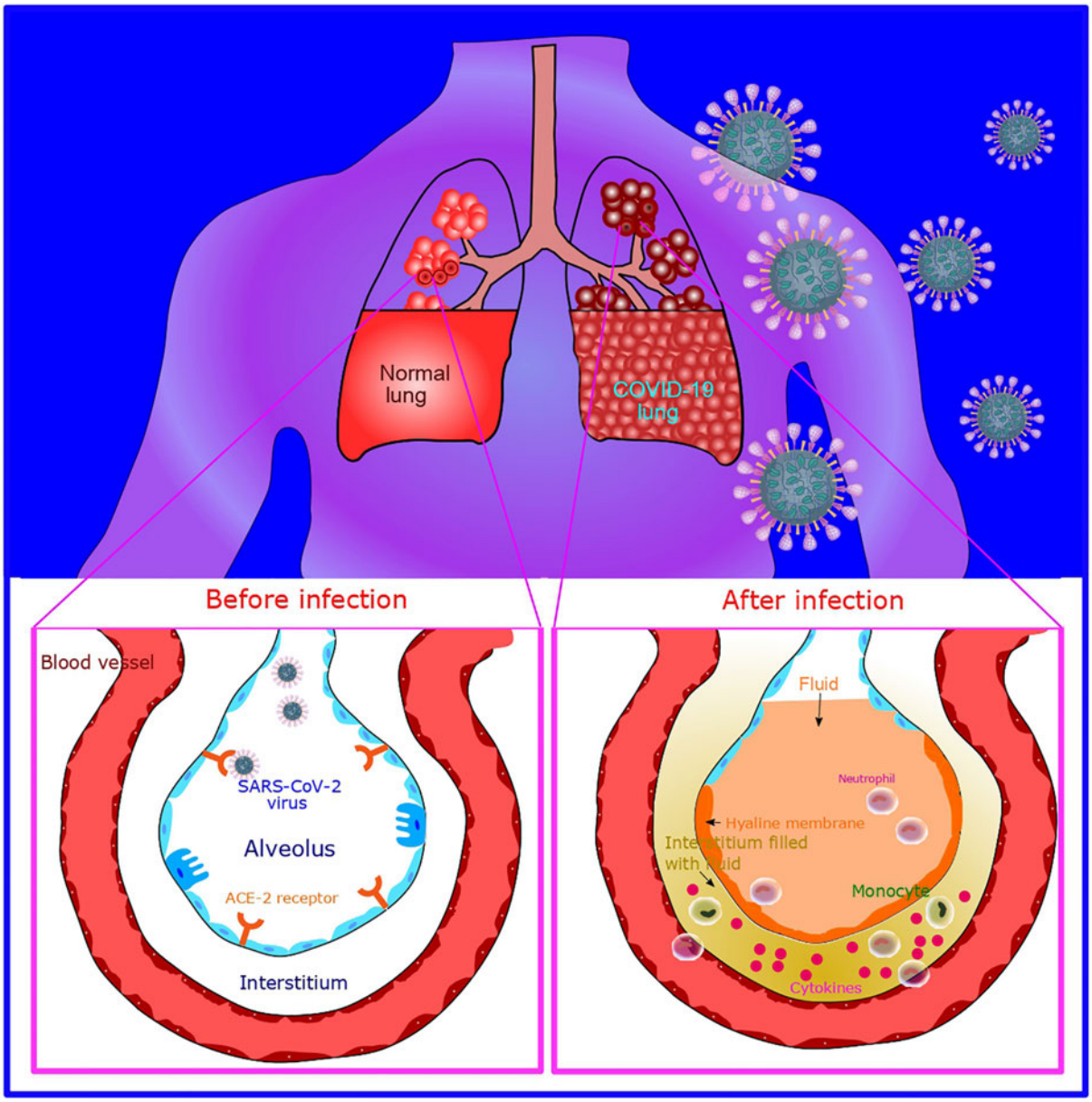


FIGURE 2 | Illustration of COVID-19 infection and its effect on the lungs. After SARS-CoV-2 infection, macrophages are released which subsequently causes cytokine release (cytokine storm). Further recruitment of neutrophills results in the release of reactive oxygen species (ROS) for destroying the infected cells. Further, fluid filling into the interstitial space and alveoli occurs.

nucleotide analog of RNA-dependent RNA polymerase (Gordon et al., 2020). An investigation in rhesus macaque model infection of MERS-CoV on preventive as well as the therapeutic potential of remdesivir revealed that the drug could reduce damage to the lungs and inhibit virus replication when administered either previous to or following infection (De Wit et al., 2020). The active metabolite of remdesivir can interact with both the active sites of enzyme and can produce delayed chain termination as well as distorted excision due to the ribose 1'-CN group, which is responsible for the enhanced antiviral action than other existing analogs (Shannon et al., 2020). Nine out of the ten ongoing clinical trials aim to evaluate the antiviral activity of remdesivir in SARS-Cov-2 infection. One of the studies is purely for the assessment of the adverse effects of the drug.

Umifenovir (Arbidol®)

Umifenovir is an antiviral agent that acts through multiple pathways and is effective against a variety of enveloped as well as non-enveloped DNA and RNA viruses. It is used for prophylaxis as well as treatment of influenza. It has been in use for more than 25 years in Russia and 14 years in China (Blaising et al., 2014). Since *in vitro* studies proved the efficacy

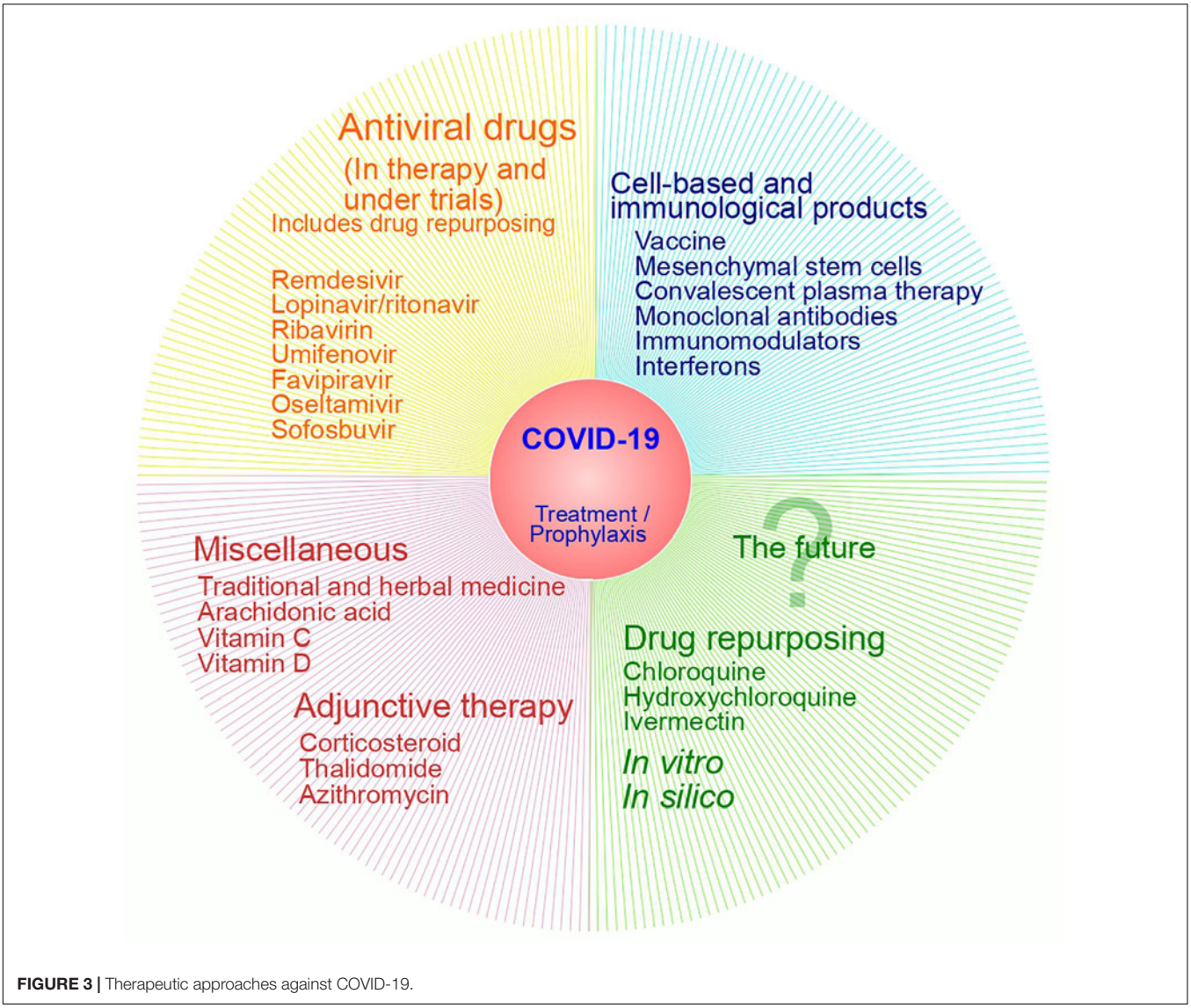


FIGURE 3 | Therapeutic approaches against COVID-19.

of this agent in SARS, it is being now used in the empirical therapy of COVID-19 in China (Song et al., 2020; Zhang J. et al., 2020). This drug is given orally for a maximum of 10 days at a dose of 200 mg, three times per day (Dong L. et al., 2020). In a study conducted in China, four patients administered with lopinavir/ritonavir, umifenovir, and one with traditional Chinese medicine along with essential support care. Three of them gained considerable relief from pneumonia, and two showed a negative viral test after the treatment period. The last patient with severe respiratory problems also showed significant improvement with this treatment procedure (Wang et al., 2020a). In a retrospective cohort study, 75% of the patients who took a combination of oral umifenovir and lopinavir/ritonavir recovered in 7 days as compared to 35% of patients who received lopinavir/ritonavir alone. And after 14 days, the viral clearance was achieved in 94% of the patients who received the drug combination, but it was only 69% in the other group (Deng et al., 2020). Also, umifenovir treatment could increase the discharging rate as well as a decline in the mortality rate(Wang et al., 2020b). Four trials are registered in ClinicalTrials.gov on umifenovir for COVID-19.

Lopinavir and Ritonavir

Both of these drugs are antiretroviral protease inhibitors and co-administration of these drugs improves the pharmacokinetics of both. Ritonavir is a potent inhibitor of microsomal enzyme cytochrome P-450 3A4, so co-administration of ritonavir leads to the increased bioavailability and half-life of the co-administered lopinavir (Cooper et al., 2003). Lopinavir/ritonavir is given two times per day in a dose of 400 mg/100 mg (Dong L. et al., 2020). After the lopinavir/ritonavir administration, coronavirus titers were null in a 54 years old male patient in Korea (Lim et al., 2020). In India, the Central Drugs Standard Control Organization agreed to use lopinavir/ritonavir therapy for 14 days with informed consent in high-risk categories who are symptomatic COVID-19 patients (Bhatnagar et al., 2020). Nevertheless, no benefit was observed in adult patients admitted

FOI 26-3132 - Document 5

TABLE 1 | Details of clinical trials on drug candidates against COVID-19.

	Intervention/treatment	Study type/Phase	Primary purpose	Number of participants	Sponsor (ClinicalTrials.gov Identifier)
1	Remdesivir	Interventional	Treatment	453	Capital Medical University (NCT04257656)
2	Remdesivir	Interventional	Treatment	308	(Capital Medical University) NCT04252664
3	Remdesivir	Expanded Access	Treatment		U.S. Army Medical Research and Development Command (NCT04302766)
4	Remdesivir	Interventional	Treatment	400	Gilead Sciences (NCT04292899)
5	Remdesivir	Interventional	Treatment	600	Gilead Sciences (NCT04292730)
6	Remdesivir	Expanded Access	Treatment	-	Gilead Sciences (NCT04323761)
7	Remdesivir	Interventional	Treatment	440	National Institute of Allergy and Infectious Diseases (NCT04280705)
8	Hydroxychloroquine	Interventional Phase 3	Treatment	440	Medical University of Vienna (NCT04336748)
9	Hydroxychloroquine Remdesivir	Interventional	Treatment	700	Oslo University Hospital (NCT04321616)
10	Chloroquine/hydroxychloroquine	Interventional Phase 2	Prevention	55000	Washington University School of Medicine (NCT04333732)
11	Chloroquine phosphate	Interventional	Treatment	250	Oxford University Clinical Research Unit, Vietnam (NCT04328493)
12	Chloroquine	Interventional Phase 2, Phase 3	Treatment	210	HaEmek Medical Center, Israel (NCT04333628)
13	Chloroquine phosphate	Interventional Phase 3	Treatment	400	Wrocław Medical University (NCT04331600)
14	Chloroquine or Hydroxychloroquine	Interventional	Prevention	4000	University of Oxford (NCT04303507)
15	Chloroquine analog, Nivolumab, Tocilizumab	Interventional Phase 2	Treatment	273	Centre Leon Berard (NCT04333914)
16	Chloroquine Diphosphate	Interventional Phase 2	Treatment	440	Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (NCT04323527)
17	Azithromycin and Chloroquine	Interventional Phase 3	Treatment	1500	Population Health Research Institute (NCT04324463)
18	Azithromycin, Hydroxychloroquine	Interventional Phase 4	Treatment	226	Chronic Obstructive Pulmonary Disease Trial Network, Denmark (NCT04322396)
19	Darunavir and hydroxychloroquine	Interventional Phase 3	Treatment	3040	Fundacio Lluita Contra la SIDA (NCT04304053)
20	Hydroxychloroquine	Interventional Phase 3	Treatment	2486	Gangnam Severance Hospital (NCT04330144)
21	Hydroxychloroquine Sulfate	Interventional	Treatment	220	University Hospital, Akershus (NCT04316377)
22	Hydroxychloroquine azithromycin	Interventional Phase 3	Treatment	440	Hospital Israelita Albert Einstein (NCT04321278)

(Continued)

FOI 26-3132 - Document 5

TABLE 1 | Continued

	Intervention/treatment	Study type/Phase	Primary purpose	Number of participants	Sponsor (ClinicalTrials.gov Identifier)
23	Hydroxychloroquine	Interventional	Treatment	1300	University Hospital, Angers (NCT04325893)
24	Levamisole, Budesonide, Formoterol, Lopinavir/Ritonavir hydroxychloroquine	Interventional Phase2 and 3	Treatment	30	Fasa University of Medical Sciences (NCT04331470)
25	Carrimycin lopinavir/ritonavir Arbidol, chloroquine phosphate	Interventional Phase 4	Treatment	520	Beijing YouAn Hospital (NCT04286503)
26	Oseltamivir, Hydroxychloroquine LopipinavirRitonavir, Darunavir Favipiravir	Interventional Phase 3	Treatment	80	Rajavithi Hospital (NCT04303299)
27	Favipiravir	Interventional	Treatment	210	Peking University First Hospital (NCT04333589)
28	Favipiravir	Interventional Phase 3	Treatment	100	Giuliano Rizzardini (NCT04336904)
29	Arbidol	Interventional Phase 4	Treatment	380	Jieming QU (NCT04260594)
30	ASC09(novel investigational protease inhibitor) lopinavir/ritonavir	Interventional	Treatment	160	First Affiliated Hospital of Zhejiang University (NCT04261907)
31	Lopinavir/ritonavir Hydroxychloroquine sulfate	Interventional phase 2	Treatment	150	Asan Medical Center (NCT04307693)
32	Lopinavir/ritonavir	Interventional phase 2	Treatment	440	Sunnybrook Health Sciences Centre (NCT04330690)
33	Hydroxychloroquine Lopinavir/ritonavir	Interventional Phase 3	Treatment	1200	Centre Hospitalier Universitaire de Saint Etienne (NCT04328285)
34	Hydroxychloroquine Oseltamivir Azithromycin	Interventional Phase 3	Treatment	500	Shehnoor Azhar (NCT04338698)
35	lopinavir/ritonavir Hydroxychloroquine Sulfate Losartan	Interventional Phase 2,3	Treatment	4000	Bassett Healthcare (NCT04328012)
36	Abidol hydrochloride, Oseltamivir Lopinavir/ritonavir	Interventional Phase 4	Treatment	400	Tongji Hospital (NCT04255017)
37	Lopinavir/ritonavir tablets Xiyanping injection	Interventional	Treatment	80	Jiangxi Qingfeng Pharmaceutical Co. Ltd. (NCT04295551)
38	lopinavir/ritonavir and Traditional Chinese Medicines	Interventional	Treatment	150	Beijing 302 Hospital (NCT04251871)
39	Methylprednisolone	Interventional Phase 2	Treatment	104	University of Trieste (NCT04323592)
40	Colchicine	Interventional Phase 3	Treatment	2500	Estudios Clínicos Latino América (NCT04328480)
41	Angiotensin 1-7	Interventional Phase 2/3	Treatment	60	Erasme University Hospital(NCT04332666)
42	Thalidomide	Interventional Phase 2	Treatment	40	First Affiliated Hospital of Wenzhou Medical University (NCT04273581)
43	Thalidomide	Interventional Phase 2	Treatment	100	First Affiliated Hospital of Wenzhou Medical University NCT04273529
44	Dietary Supplement: Natural Honey	Interventional Phase 3	Treatment	1000	Misr University for Science and Technology (NCT04324489)

in the hospital due to severe COVID-19 with lopinavir/ritonavir therapy beyond standard care (Cao et al., 2020). More similar remarks were noticed and accordingly, the benefits of this combination are still doubtful (Kupferschmidt and Cohen, 2020). As a result, experts opined that the effectiveness of remdesivir and lopinavir/ritonavir should be confirmed by a randomized controlled trial. A retrospective data of pediatric patients with confirmed COVID-19 shows that all 36 children received interferon-alfa (INFα), 14 received lopinavir/ritonavir, and 6 needed oxygen inhalation resulted in full recovery irrespective of the drug within 14 ± 3 days. Treatment with INFα along with lopinavir/ritonavir plus ribavirin showed a beneficial action in COVID-19 therapy (Yuan et al., 2020). At the same time, some reports expressed that the use of lopinavir/ritonavir along with adjuvant drugs should be encouraged for the treatment of patients with COVID-19 (Ye et al., 2020). More than 20 clinical trials are registered for the evaluation of this combination in COVID-19.

Favipiravir

Favipiravir, a derivative of pyrazine carboxamide, is a purine nucleic acid analog that interferes with the replication of the virus and inhibits RNA dependent RNA polymerase of RNA viruses. It possesses broad-spectrum antiviral activity and is effective against the influenza virus, bunyavirus, arenavirus, and filovirus (Du and Chen, 2020; Singh et al., 2020). An early result of a clinical trial reveals that favipiravir has more strong anti-viral activity than lopinavir/ritonavir with significantly less adverse effects (Chavez et al., in press; Zhai et al., 2020). Due to its efficacy on virus clearance, the Turkish ministry of health approved favipiravir for treating critical patients with Covid-19 pneumonia (Kodaz, 2020). Eleven clinical trials are registered for this drug in COVID-19.

Oseltamivir

It is a neuraminidase inhibitor that hinders the neuraminidase enzyme expressed on the surface of the virus. This enzyme is needed for the release of the virus from the cells. It is approved for the prophylaxis as well as for the treatment of influenza (Li et al., 2012). Five trials are registered for this drug in COVID-19 treatment and details are given in **Table 1**.

In the study by Ding et al. about the clinical characteristics of COVID-19 patients, all the 115 subjects received oseltamivir along with antibiotics and oxygen inhalation and all recovered without the need for intensive care unit (ICU; Ding et al., 2020). A 71-year-old woman tested positive for COVID-19 with a childhood history of psoriasis pointed out exacerbation of psoriasis after oseltamivir and hydroxychloroquine treatment. Hydroxychloroquine inhibits epidermal transglutaminase, which leads to the collection of the epidermal cells, and to date, there were no reports that oseltamivir may affect psoriasis. Thus, it could be reasonably argued that hydroxychloroquine may lead to a global increase in the number of psoriasis (Kutlu and Metin, 2020).

In the study conducted by Huang et al. (2020), all the 41 patients were given empirical antibiotic treatment, and 93% received oseltamivir also, but the results are yet to be known. A 43-year-old female patient was recovered and discharged from the hospital after the use of oseltamivir along with traditional Chinese medicine. Unfortunately, this patient showed a positive SARS-CoV-2 test again after 22 days of hospital discharge, however, convalescent plasma therapy (CPT) along with other measures made her condition better (Chen D. et al., 2020; Luo, 2020).

Ribavirin

Ribavirin is a guanosine analog and nucleoside inhibitor to stop viral RNA synthesis. It was widely used to treat SARS in combination with or without steroids in severe cases. Virtual screening of some FDA approved medicines against SARS-CoV-2 main protease (M^{pro}) has been done and found that ribavirin, telbivudine, vitamin B12, and nicotinamide has an excellent docking score and can be made use in the treatment of COVID-19 (Kandeel and Al-Nazawi, 2020). In another molecular docking study, it was found that ribavirin, galidesivir, sofosbuvir, remdesivir, and tenofovir are effective agents in the treatment of COVID-19 since these drugs can tightly bind to the viral RNA dependent RNA polymerase (Elfiky, 2020). Since ribavirin shows the adverse effect of decreasing hemoglobin levels, it is not recommended for patients with respiratory distress (Jean et al., 2020). Out of the two clinical trials registered for this drug, one is completed, but results are not yet available.

Cell-Based and Immunological Products

Monoclonal Antibodies

In COVID-19 infection, activation of a huge number of mononuclear macrophages and T lymphocytes occur which results in the production of cytokines such as IL-6. This IL-6 will bind to the IL-6 receptor on the target cells, which leads to cytokine storm as well as dangerous inflammatory responses in the lungs and other organs (Xu et al., 2020).

Tocilizumab, a humanized monoclonal antibody that acts as a blocker of the IL-6 receptor, can bind to the IL-6 receptor with high affinity. It can prevent IL-6 itself from binding to its receptor, making it unable to injure the target cells, and lessen the inflammatory responses (Zhang C. et al., 2020). Its use is considered as one of the latest treatment strategies against COVID-19 (Bersanelli, 2020). The results of a retrospective study in fifteen COVID-19 patients, including moderate to critically ill, suggests that tocilizumab can be an efficient management option for patients with a risk of cytokine storms (Luo et al., 2020). Treatment with Tocilizumab, which blocks IL-6 receptors, results in notable outcomes like reduction in the elevated body temperature and improved respiratory function (Fu et al., 2020). Tocilizumab is also referred to as a promising choice for the treatment of the hyperinflammatory state associated with this infection in the second edition of "Vademecum for the Treatment of People With COVID-19" (Lombardy Section Italian Society Infectious And Tropical Diseases, 2020). In a COVID-19 patient with multiple myeloma, the treatment with tocilizumab was successful. The study recommends the need for randomized clinical trials for detailed evidence (Zhang X. et al., 2020). Further, several studies have been reported the use of tocilizumab as a promising treatment option for COVID-19 related respiratory

failure (Bachanova et al., 2020; Bennardo et al., 2020; Buonaguro et al., 2020; Cellina et al., 2020; Ceribelli et al., 2020; Liu B. et al., 2020; Michot et al., 2020; Mihai et al., 2020).

Coronavirus neutralizing antibodies mainly aim the spike proteins on the surface of the virus, which mediate the entry into host cells. Receptor binding can generate irreversible conformational alteration in the spike proteins and thus inhibits the viral fusion with host cells (Wang C. et al., 2020). It is now suggested that CR3022, a SARS-CoV-specific human monoclonal antibody, has the potential to emerge as an agent against SARS-CoV-2, along with other neutralizing antibodies (Tian et al., 2020). Sarilumab, gimsilumab, lenzilumab, etc. are the other monoclonal antibodies in the trial. More than 40 clinical trials on monoclonal antibodies are registered, including those on tocilizumab, sarilumab, gimsilumab, and lenzilumab. Details of selected clinical trials on biological agents against COVID-19 are provided in **Table 2**.

Interferons

Interferons are signaling proteins and have antiviral activity. Viruses trigger the release of interferons by the host cells. Type 1 interferons possess a wide range of antiviral effects *in vitro* and a recent clinical trial proved its efficacy in the treatment of MERS-CoV. With regard to this, interferon wastried in clinical trials as a treatment option for COVID-19. The β subtype is found more promising, and the treatment in the early stages of the infection is recommended (Sallard et al., 2020). The combination of ribavirin and INFα has been the most commonly used therapy to treat MERS outbreaks in South Korea (Kim et al., 2016). Due to the effectiveness of this therapy, this combination is recommended to treat COVID-19 infection in the fifth edition of the National Health Commission's Regimen of China (Du et al., 2020).

Mesenchymal Stem Cells

The human umbilical cord mesenchymal stem cell (MSC) possesses outstanding immunomodulatory and strong anti-inflammatory functions with proper safety (Metcalfe, 2020). Liang et al. (2020) reported that treatment with allogeneic human umbilical cord MSCs in a 65-year-old critically ill female patient with COVID-19 showed a significant and positive outcome with good tolerance. So this kind of therapy is an ideal choice for the management of seriously ill COVID-19 patients (Liang et al., 2020). In another study, seven patients who are dangerously ill with COVID-19, MSCs therapy significantly improved their condition without any adverse effects (Leng et al., 2020). Therefore, MSC therapy is a safe and effective option for critical cases of pneumonia associated with COVID-19. Even though the results are promising, proper clinical investigations are required for these kinds of cell-based therapies (Khoury et al., 2020). A total of 29 clinical trials are registered for the efficacy and safety evaluation of MSCs in COVID-19.

Convalescent Plasma Therapy

Convalescent plasma therapy is considered one of the advanced options sought for the treatment of COVID-19 (Zhao and He, 2020). Convalescent or immune plasma is the plasma collected from individuals who are cleared of infection with a sufficient amount of developed and antibodies. Convalescent plasma therapy will help to get immediate immunity for a short time in susceptible individuals (Bloch et al., 2020). Neutralizing antibodies are critical in virus clearance and vital in defense against various viral diseases. Passive immunity achieved due to convalescent plasma can provide neutralizing antibodies that can control the infection. In addition to antiviral action, convalescent plasma can also cause immunomodulation. Some antibodies can cause the inhibition of complement cascade and thus can control the formation of immune complexes. Convalescent plasma has anti-inflammatory effects also due to the action of a network of autoantibodies and can manage an overactive immune system. The effectiveness of this therapy is highly related to the concentration of neutralizing antibodies in the collected plasma (Rojas et al., 2020). A brief mechanism of CPT is depicted in **Figure 4**.

Despite this apparent advantage, several challenges are also associated with CPT such as anaphylactic reactions, transfusion transmitted infections, transfusion associated acute lung injury, circulatory overload, hemolysis, etc. Owing to the importance of this approach, the possibilities and challenges of CPT are now well discussed and described (Roback and Guarner, 2020).

From the previous studies related to SARS, it was reported that CPTresulted in a shorter hospital stay period as well as a lower death rate as compared to the control group (Soo et al., 2004; Cheng et al., 2005; Chen L. et al., 2020). Five seriously sick patients with COVID-19 showed promising results when received plasma transfusion. After the transfusion, body temperature and viral loads were declined and turned to negative within 12 days (Shen et al., 2020). In another study, a single dose of 200 mL of convalescent plasma antibody titers over 1:640 was transfused to 10 patients along with antiviral agents and supportive measures. The results confirmed that this therapy was well tolerated and could be able to neutralize viremia in critical COVID-19 cases (Duan et al., 2020).

In April 2020, the US FDA permitted CPT in dangerously sick patients with COVID-19 (Tanne, 2020). Two COVID-19 patients with severe pneumonia and ARDS showed a positive result by CPT along with corticosteroids (Ahn et al., 2020). A good number of clinical trials are registered for studying the safety and efficacy evaluation of CPT in COVID-19 patients (**Table 3**). Many clinical results showed that CPT produces remarkable improvement in clinical symptoms as well as radiological and biochemical parameters related with the SARS-CoV-2 infection (Bakhtawar et al., 2020).

Vaccines

Vaccine development needs many years to reach the market under normal circumstances. Fortunately, the data generated in the research on SARS-CoV as well as MERS-CoV helped in an express design and development of the COVID-19 vaccine. Thus, within three months of the emergence of COVID-19, a specific vaccine candidate entered Phase I clinical trials, and WHO announced the news of the availability of licensed vaccine for widespread use by the middle of 2021. Presently, different SARS-CoV and MERS-CoV vaccine candidate are in clinical trials against COVID-19. At the same time, thorough investigations

TABLE 2 | Details of clinical trials on biological agents against COVID-19.

No.	Intervention/treatment	Study type/Phase	Primary purpose	No. of participants	Sponsors and Collaborators (ClinicalTrials.gov Identifier)
1	Lopinavir/ritonavir Hydroxychloroquine sulfate Baricitinib (Janus kinase inhibitor) Sarilumab (anti-IL-6 receptor)	Interventional (Phase 2)	Treatment	1000	Lisa Barrett, Nova Scotia Health Authority (NCT04321993)
2	Xiyanping injection Lopinavir/ritonavir, alpha-interferon nebulization	Interventional (Phase 2,3)	Treatment	348	Jiangxi Qingfeng Pharmaceutical Co. Ltd. (NCT04275388)
3	Remdesivir Lopinavir/ritonavir, Interferon Beta-1A, Hydroxychloroquine	Interventional	Treatment	3100	Institut National de la Santé Et de la Recherche Médicale, France (NCT04315948)
4	lopinavir/ritonavir, remdesivir, interferon beta-1a, chloroquine and/or azithromycin	Observational	Adverse events	1000	Groupe Hospitalier Pitie-Salpetriere (NCT04314817)
5	Hydrocortisone, Ceftriaxone, Moxifloxacin, Levofloxacin, Piperacillin-tazobactam, Ceftaroline, Amoxicillin, clavulanate, Macrolide, oseltamivir, Lopinavir/ritonavir Hydroxychloroquine Interferon-β1a Anakinra	Interventional	Treatment (Phase 4)	6800	MJM Bonten (NCT02735707)
6	Lopinavir/ritonavir, Ribavirin, Interferon Beta-1B	Interventional (Phase 2)	Treatment	70	The University of Hong Kong (NCT04276688)
7	ASC09F Oseltamivir Ritonavir Oseltamivir	Interventional (Phase 3)	Treatment	60	Tongji Hospital (NCT04261270)
8	Hydroxychloroquine Lopinavir/Ritonavir Interferon Beta-1A Interferon Beta-1B	Interventional (Phase 4)	Treatment	60	Shahid Beheshti University of Medical Sciences
9	Abidol Hydrochloride Interferon	Interventional (Phase 4)	Treatment	100	Tongji Hospital (NCT04254874)
10	Recombinant human interferon Alpha-1b thymosin alpha 1	Interventional (Phase 3)	prevention	2944	Shanghai Jiao Tong University School of Medicine (NCT04320238)
11	Ganovo, ritonavir, Interferon	Interventional (Phase 4)	Treatment	11	The Ninth Hospital of Nanchang (NCT04291729)
12	Recombinant human interferon α1β	Interventional (early Phase 1)	Treatment	328	Tongji Hospital (NCT04293887)
13	Bromhexine Hydrochloride, Arbidol Hydrochloride Recombinant Human Interferon α2b	Interventional	Treatment	60	Second Affiliated Hospital of Wenzhou Medical University (NCT04273763)
14	Emapalumab(Anti-interferon Gamma) Anakinra (Interleukin-1Receptor Antagonist)	Interventional (Phase 2/3)	Treatment	54	Swedish Orphan Biovitrum (NCT04324021)
15	Tocilizumab	Observational	Treatment	30	University of L'Aquila (NCT04332913)
16	Favipiravir Tocilizumab	Interventional	Treatment	150	Peking University First Hospital (NCT04310228)
17	INO-4800, a Prophylactic Vaccine	Interventional (non-randomized)	Prevention	40	Inovio Pharmaceuticals (NCT04336410)
18	Biological: UC-MSCs	Interventional	Prevention	10	ZhiYong Peng (NCT0426952)
19	Biological: ChAdOx1 nCoV-19	Interventional (Phase 1/2)	Treatment	510	University of Oxford (NCT04324606)
20	Tocilizumab Sarilumab	Interventional (Phase 2)	Treatment	200	Marius Henriksen (NCT04322773)
21	Siltuximab Methylprednisolone	Interventional (Phase 2)	Treatment	100	Judit Pich Martínez (NCT04329650)
22	Tocilizumab Pembrolizumab (MK-3475)	Interventional (Phase 2)	Treatment	24	MedSIR (NCT04335305)

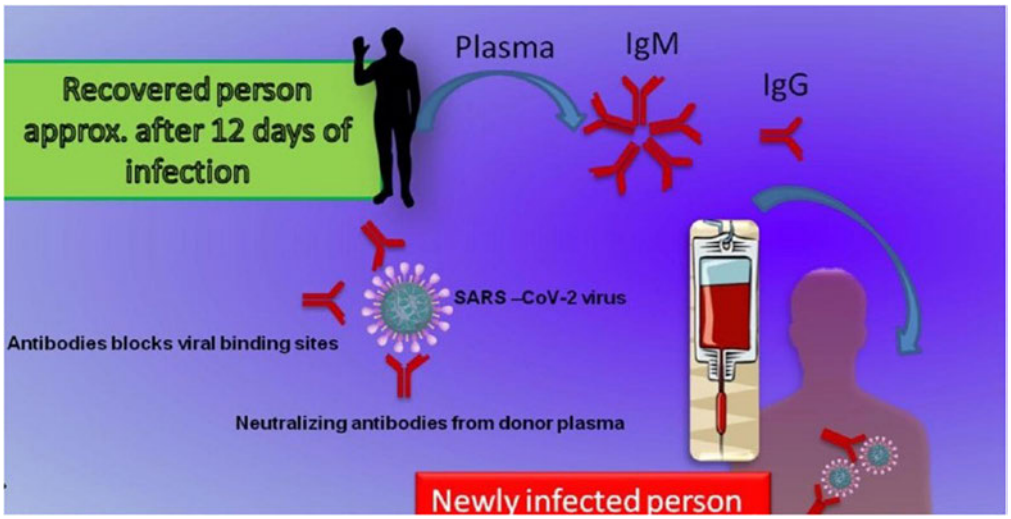


FIGURE 4 | Schematic representation of the convalescent plasma therapy along with its mechanisms of action. A person recovered from COVID-19 infection produces a sufficient amount of specific antibodies in 12–14 days. The plasma with neutralizing antibodies mainly IgM and IgG can be transferred to produce immediate immunity in suspected or infected persons. IgG and IgM anti-SARS-CoV-2 antibodies will bind to specific sites and neutralize the virus.

are required urgently to study the risk of immune enhancement. Therefore, along with early clinical trials, clinical evidence is also needed to support the possibility of immune enhancement (de Alwis et al., 2020). According to the latest WHO report there are more than 169 COVID-19 vaccine candidates currently under clinical trials. Among these clinical candidates, 26 are in the various phase of human trials and 10 reached up to phase 3 of the clinical trial. **Figure 5** shows some of the ongoing vaccine trials in lead which are specific to COVID-19.

Miscellaneous/Adjunctive Therapy

Traditional Chinese Medicine

Various Traditional Chinese Medicines like Shuanghuanglian oral liquid and Lianhuaqingwen capsule were also tried for treating SARS-CoV-2 infection and found satisfactory outcome. Some of them, like YinHu QingWen, Fuzheng Huayu, are in registered clinical trials (Wang L. et al., 2020). A total of 8 clinical trials are registered for the evaluation of the effectiveness of Traditional Chinese Medicine.

Vitamins

An adequate level of vitamin D is required to suppress the adhesion molecule (CD26), which helps in invasion to host cells. Since, vitamin D deficiency leads to increased risk of respiratory infections, supplementation with adequate dose is highly recommended in high-risk categories (McCartney and Byrne, 2020). Clinical studies show that a large dose of vitamin C can prevent viral infections. Thus, early use of antioxidants like vitamin C in large doses may be helpful to fight COVID-19 (Cheng, 2020).

Corticosteroids

Even though corticosteroids are used as adjuvant therapy in COVID-19 infection, WHO and the United States Centre for Disease Control and Prevention recommend that it should not be regularly used in COVID-19 patients, except indicated for other conditions. Low to moderate doses with close monitoring as a short course may be beneficial. More than ten trials are registered for the evaluation or comparison of the safety as well as efficacy of different corticosteroids in COVID-19 (Rosa and Santos, 2020).

Thalidomide

The severe lung injury in COVID 19 may be related to the extreme immune response as a result of cytokine storm. Thalidomide is an immunomodulatory agent, and it can stimulate T cells, decrease TNF-α production, and enhance the secretion of interleukins and natural killer cells. Its anti-inflammatory role is due to the capacity to reduce TNF-α by enhancing the degradation of mRNA in blood cells. Two trials are registered to test the safety and effectiveness of thalidomide in treating moderate or severe COVID-19 (Newfield, 2018).

Drug Repurposing Approach

Chloroquine or Hydroxychloroquine

Chloroquine is a popular drug used for the prophylaxis of malaria and amebiasis and also to treat autoimmune diseases like rheumatoid arthritis and lupus erythematosus. A wide range of mechanisms has been proposed for the action of chloroquine (Cortegiani et al., 2020). By altering the binding of the virus to the cell surface receptor, it can hold back the pre-entry step to the host cell (Vincent et al., 2005). Also, it can impair pH reliant endosome dependent entrance of enveloped viruses like chikungunya and dengue viruses (Gay et al., 2012; Wong and Chu, 2018). Post-translational variation of viral proteins can also be damaged by chloroquine. It can also spoil the viral protein maturation (Devaux et al., 2020). In addition, it acts as an immunomodulatory agent by the regulation of pro-inflammatory cytokines and cell signaling.

FOI 26-3132 - Document 5

TABLE 3 | Details of convalescent plasma therapies at clinical trials.

No.	Intervention/treatment	Study type/Phase	Primary purpose	Number of participants	Sponsors and Collaborators (ClinicalTrials.gov Identifier)
1	Convalescent Plasma	Interventional (Phase 1)	Treatment	20	Hospital San Jose Tec de Monterrey (NCT04333355)
2	Anti-SARS-CoV-2 convalescent plasma	Interventional (Early Phase 1)	Treatment	20	Orthosera Kft. (NCT04345679)
3	Convalescent Plasma Transfusion	Interventional (Phase 2)	Treatment	20	Institute of Liver and Biliary Sciences, India (NCT04346446)
4	Convalescent Plasma	Interventional (Phase 2)	Treatment	15	Saint Francis Care (NCT04343261
5	COVID-19 convalescent plasma	Expanded Access	Treatment	Expanded Access	Mayo Clinic (NCT04338360)
6	Transfusion of COVID-19 convalescent plasma	Interventional (Phase 2)	Treatment	120	Assistance Publique - Hôpitaux de Paris (NCT04345991)
7	convalescent plasma from recovered COVID 19 donor	Interventional (Phase 2)	Treatment	40	King Fahad Specialist Hospital Dammam (NCT04347681)
8	Convalescent Plasma Standard Donor Plasma	Interventional (Phase 1/2)	Treatment	500	Stony Brook University (NCT04344535)
9	Convalescent Plasma	Interventional (Phase 2)	Treatment	55	Hackensack Meridian Health (NCT04343755)
10	Convalescent Plasma	Interventional (Phase 2)	Treatment	426	Erasmus Medical Center (NCT04342182)
11	Convalescent Plasma	Interventional (Phase 2)	Treatment	278	Cristina Avendaño Solá (NCT04345523)
12	Convalescent Plasma	Interventional (EarlyPhase1)	Treatment	10	University of Chicago (NCT04340050)
13	Plasma Hydroxychloroquine Azithromycin	Interventional (Phase 1/2)	Treatment	80	Universidad del Rosario (NCT04332835)
14	Convalescent Plasma	Interventional	Treatment	30	Mazandaran University of Medical Sciences (NCT04327349)
15	Convalescent Plasma	Observational		15	Shanghai Public Health Clinical Center (NCT04292340)
16	Anti- SARS-CoV-2 Plasma SARS-CoV-2 non-immune Plasma	Interventional (Phase 2)	Treatment	150	Johns Hopkins University (NCT04323800)
17	Convalescent anti-SARS-CoV-2 plasma Sarilumab, Baricitinib, Hydroxychloroquine	Interventional (Phase 3)	Treatment	1500	Thomas Benfield (NCT04345289)
18	high-titer anti-Sars-CoV-2 plasma, oxygen therapy	Interventional (Phase 1)	Treatment	115	Baylor Research Institute (NCT04333251)
19	Anti-coronavirus antibodies (immunoglobulins)obtained with DFPP from a convalescent patient	Interventional	Treatment	10	A.O. Ospedale Papa Giovanni XXIII (NCT04346589

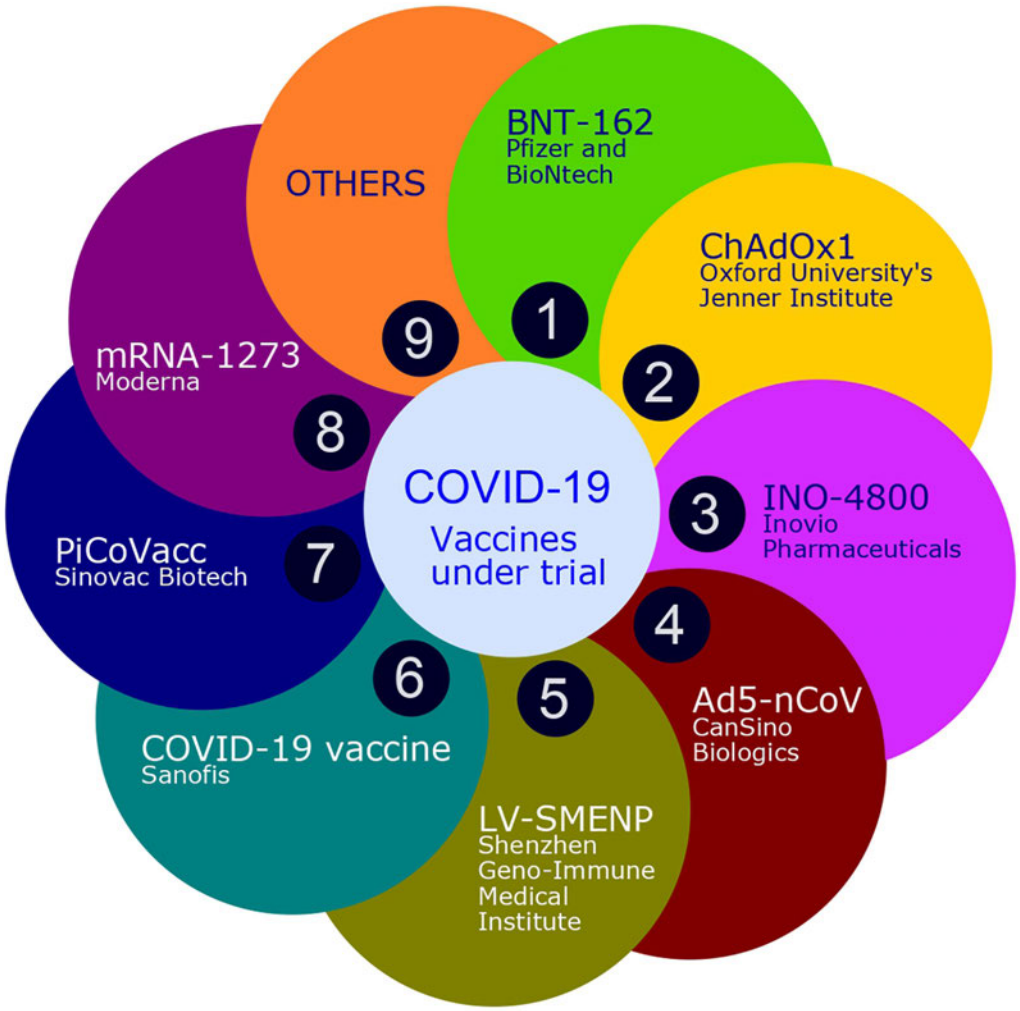


FIGURE 5 | Some of the ongoing vaccine trials in lead which are specific to COVID-19.

Several trials on the effectiveness of this agent have been found to be registered in the Chinese Clinical Trial Registry. Based on this, Liu and associates examined the antiviral action of hydroxychloroquine and chloroquine against COVID-19 *in vitro*. It was found that both the agents elevate the intracellular organelles' pH, which is critical in membrane fusion. Both the drugs were able to exert their actions to inhibit the viral entry as well as at some post-entry stages. It was found that the drugs could block the transport of the virus from the early endosomes to endolysosomes, which is essential for the release of the viral genome. The study concludes that hydroxychloroquine is as effective as chloroquine for the *in vitro* inhibition of SARS-CoV-2 infection with comparatively less toxicity (Liu J. et al., 2020). A survey in a University Hospital in Marseille reassured the disappearance or decrease of viral load in hydroxychloroquine treated patients, and the result is reinforced by concomitant use of azithromycin (Gautret et al., 2020).

Wang and associates demonstrated the effectiveness of chloroquine at entry as well as post-entry phases of COVID-19 infection *in vitro* cell lines. Further, the immune modulation property can induce synergism in its antiviral activity *in vivo* (Wang M. et al., 2020). Still, there is an opinion that the option of using chloroquine for the treatment of COVID-19 should be properly analyzed in light of the new hopeful declarations, by considering the possible side effects (Colson et al., 2020; Touret and de Lamballerie, 2020). Chloroquine causes under expression of phosphatidylinositol binding clathrin assembly protein and thereby affects endocytosis (Hu et al., 2020). Meanwhile, the assessment of safety and efficiency of chloroquine for treating COVID-19 demands the need for safety data from urgent high-quality trials from various geographical areas (Cortegiani et al., 2020). Interestingly, chloroquine phosphate shortened the course of the disease in clinical trials and thus seems to be better than the control, for the inhibition of pneumonia (Gao et al., 2020) Also, *in vitro* study revealed that hydroxychloroquine shows the more powerful effect as compared to chloroquine (Yao et al., 2020). As of now, more than 40 clinical trials are registered to assess and/or

compare the effectiveness and/or safety of chloroquine and hydroxychloroquine.

Metronidazole

Metronidazole is another nucleic acid synthesis inhibitor and a potential candidate which can counter most of the immunopathological symptoms of SARS-CoV-2 infection. *In vitro* as well as *in vivo* studies proved that this drug could reduce cytokine levels, which are generally increased during this disease. *In vitro* studies proved that metronidazole at high doses had a marked inhibitory effect on lymphoproliferative assay (Fararjeh et al., 2008; Rizzo et al., 2010). Also, it can reduce neutrophil-generated ROS in the event of inflammation. But studies with a big number of groups are needed to prove its effectiveness (Gharebaghi et al., 2020)

Sofosbuvir, an FDA approved nucleotide polymerase inhibitor mainly used for the management of hepatitis C is under test by a Chinese research foundation (Vellingiri et al., 2020). Previously sofosbuvir was used along with ribavirin and interferon. The use of sofosbuvir is reported in the management of the Zika virus also (Cheema et al., 2019).

Some Other Potential Agents

Baricitinib, carfilzomib, indinavir, baloxavir, ruxolitinib, fedratinib, and azvudine are some other potential agents to treat this respiratory disease (Peter et al., 2020). Among these, fedratinib, baricitinib, and ruxolitinib are potent anti-inflammatory agents and powerful Janus kinase inhibitors (Stebbing et al., 2020) which are approved for rheumatoid arthritis and myelofibrosis. It is reported that these drugs are effective to control the increased levels of cytokines usually observed in COVID-19 patients (Stebbing et al., 2020). Twelve clinical trials have been registered to evaluate the efficacy and safety of ruxolitinib in COVID-19 patients. Baricitinib is not considered an ideal choice for the treatment of COVID-19, since it may enhance the chance of co-infection and increase the incidence of anemia (Praveen et al., 2020). Eight clinical trials are registered for the evaluation of the efficacy of baricitinib in COVID-19 patients. The immune-modulating and anti-inflammatory agents are not usually recommended in pneumonia associated with COVID-19. Nevertheless, as per the pathology of pulmonary edema as well as the formation of hyaline membrane, it could be reasonably assumed that well-timed and appropriate therapy with immunomodulators along with other supportive measures may save the COVID-19 patients from ARDS. Based on these considerations, one clinical trial is going on for the evaluation of fingolimod, an immunology modulator generally used in multiple sclerosis (ClinicalTrails, 2020).

Nitazoxanide is an antiprotozoal drug with a broad-spectrum antiviral activity, which can also reduce the production of pro-inflammatory cytokines (Kelleni, 2020). With regard to this feature, it has been registered for studies in more than 6 clinical trials as monotherapy or as combination therapy for the management of COVID-19 (ClinicalTrails, 2020).

Severe acute respiratory syndrome coronavirus-2 infection depends on ACE2 and TMPRSS2 proteins on the host cell surface and these can be blocked by protease inhibitors (Nadeem et al., 2020). Camostat mesylate is a synthetic serine protease inhibitor that can inhibit TMPRSS2 protein in lung cells (Hoffmann et al., 2020; McKee et al., 2020). Nafamostat mesylate, another TMPRSS2 serine protease inhibitor, may also prevent the entry of virus into the host cell. Cell culture experiments proved that nafamostat mesylate inhibited SARS-CoV-2 infection in SARS-CoV-2 infected Vero E6 cells (EC_{50} = 22.5 μM) (Wang M. et al., 2020). There are numerous similarities in clinical, pathological, and laboratory findings of moderate to severe SARS-CoV-2 infection and haemophagocytic lymphohistiocytosis. The possibility of etoposide may be considered for treating haemophagocytic lymphohistiocytosis associated with moderately severe or severe forms of SARS-CoV-2 infection (Hamizi et al., 2020). The viroporine channel of COVID-19 can be effectively blocked by amantadine and thus can prevent viral genome release into the cytoplasm. Therefore, amantadine can be used to mitigate the effects of COVID-19 if used in an early stage of infection (Abreu et al., 2020). Niclosamide is another potential option since it can block endocytosis and autophagy of SARS-CoV-2 (Pindiprolu and Pindiprolu, 2020).

Drug repurposing approach by *in silico* studies will give potential clues about the agents which might be helpful to fight this deadly virus. It was found that lopinavir, galidesivir, asunaprevir, CGP42112A, remdesivir, indinavir, ABT450, ritonavir, and methisazone can interact with more than two protein structures of COVID-19. Among these, HIV protease inhibitors exhibited excellent outcomes in docking studies (Shah et al., 2020). Similarly, Wu and associates carried out an analysis by computational methods for discovering therapeutic targets of novel coronavirus as well as predicting potential medicines. The study reports more than 50 natural compounds and more than 50 drugs which may be considered for further studies for the treatment of COVID 19 (Wu et al., 2020). In a study using the drug-target interaction model, atazanavir, remdesivir, efavirenz, ritonavir, dolutegravir, lopinavir, darunavir showed good inhibitory potency, with atazanavir in the first position, followed by remdesivir (Beck et al., 2020). When docking was performed with solvent molecular dynamics on several natural compounds, compounds like 5,7,3',4'-Tetrahydroxy-2'-(3,3-dimethylallyl) isoflavone, myricitrin, and methyl rosmarinate were observed to be the most promising agents against COVID-19 (ul Qamar et al., 2020). Muralidharan et al. conducted computational studies to understand the synergism of lopinavir, ritonavir, and oseltamivir. They found that the combination of these three drugs resulted in superior binding energy as compared to the individual agents (Muralidharan et al., 2020).

Based on Feline Infectious Peritonitis Strategies, Olsen et al. (2020) suggested the use of nelfinavir and amodiaquine for the treatment of SARS-CoV-2 infection with a potential CNS invasion. *In vitro* study in Vero-E6 cells showed that remdesivir and lopinavir inhibit the replication of SARS-CoV-2 with EC50 at 23.15 and 26.63 μM concentrations, respectively. At the same time, homoharringtonine and emetine have EC50 values of 2.55 and 0.46 μM, respectively, for the inhibition of SARS-CoV-2 replication. In addition, a synergistic effect

for the combination of remdesivir and emetine has been also observed (Choy et al., 2020). It may be noted that a 60-year-old immunocompromised female cancer patient on darunavir/cobicistat along with hydroxychloroquine was able to leave the hospital within six days, which shows the potential of darunavir/cobicistat even in immunocompromised patients (Spezzani et al., 2020).

RAC/CDC42-activated kinases (PAK1) are required for the pathogenic process of different kinds of viruses including SARS-CoV-2. Therefore, PAK1 blockers like melatonin, propolis, cicloresonide, some anti-malaria drugs like ivermectin, and ketorolac could act as promising agents against COVID-19 (Maruta and He, 2020). Ivermectin is a broad-spectrum anti-parasitic agent approved by the FDA. An *in vitro* study demonstrated that it can cause a 5000 times decrease in SARS-CoV-2 viral RNA in 48 h and it needs further investigation and clinical trials as a promising therapeutic agent against COVID-19 (Caly et al., 2020). This study received wide attention which finally led USFDA to issue a letter clarifying that the study tested ivermectin neither in humans or animals. The letter also warned the use of ivermectin containing veterinary products in humans (FDA, 2020). The mechanism of action and dose of some potential drugs against COVID-19 are given in **Table 4**.

PERSPECTIVES

Hopefully, several perspectives and hypotheses are available related to the treatment and prophylaxis against COVID-19. Some of them appear interesting and promising for further consideration and studies. In such a perspective, it is mentioned that ATP has a crucial role in cellular function and cyclic ATP depletion can cause cellular dysfunction including immune cells. ATP-repletion can prevent the "cytokine storm" in COVID-19 and increase the cellular energy to fight against the virus (Taghizadeh-Hesary and Akbari, 2020). Other than the antiviral activity chloroquine/hydroxychloroquine cause the movement of extracellular zinc into intracellular lysosomes and thus hinders the enzyme RNA polymerase which is required for viral replication. Zinc deficiency is commonly seen in geriatrics and also in patients with diabetes, chronic pulmonary disease, cardiovascular diseases, etc. Therefore, zinc supplementation along with chloroquine therapy may reduce the mortality and morbidity rate in COVID-19 (Derwand and Scholz, 2020). Copper is a very important micronutrient required for the functioning of vital immune cells like B cells, helper T cells, natural killer cells, macrophages, etc. Therefore, enhancement of plasma level of copper may boost the immune system and may act as a preventive or therapeutic measure against COVID-19 (Raha et al., 2020). If COVID-19 results in long term cardiopulmonary damage, cardiopulmonary rehabilitation is required; exercise can be considered as a therapy of choice. Since physical movement of many people has become less in this pandemic situation, therefore exercise should be given prime importance. It is proposed that exercise may help in lowering the risk of SARS-CoV-2 infection by minimizing cardiopulmonary sequel in the recovery period (Heffernan and Young Jae, 2020). In a hypothesis, it is postulated that the

TABLE 4 | The mechanism of action and dose of potential drugs against COVID-19.

Drug	Mechanism of action	Dose
Remdesivir	Inhibition of RNA polymerase	200 mg initial dose after that 100 mg daily (IV) up to 9 days
Chloroquine/ Hydroxychloroquine	Modify the transcription process and signaling pathways	400 mg two times on the first day, then 200 mg two times up to 7 days
Umifenovir	Inhibition of membrane fusion	200 mg three times daily maximum up to10 days
Lopinavir/Ritonavir	Protease inhibitor	400/100 mg two times a day for 14 days
Favipiravir	Inhibits viral replication	1600 mg two times in the first day, then 600 mg two times per day up to 6 days
Oseltamivir	Reduce viral replication	75 mg two times daily up to 5 days
Ribavirin	Nucleoside inhibitor	500 mg two or three times daily along with interferon α or lopinavir/ritonavir maximum up to10 days
Metronidazole	Nucleic acid synthesis inhibitor	400 mg two times daily maximum up to 14 days
Baricitinib	Anti-Janus kinase inhibitor	4 mg/day for two weeks
Camostat Mesilate	Block cell entry (Serine protease inhibitor)	200 mg three times daily for 5 days
Darunavir/Cobicistat	Protease inhibitor/inhibitor of cytochrome P450 3A	800 mg/150 mg once daily for 5 days
Thalidomide	Anti-inflammatory, anti-angiogenesis, antifibrotic and immune regulation	100 mg for 14 days
Isotretinoin	Down regulator of ACE-2 receptors also PLpro inhibitor	0.5 mg per kg daily for one month
IFN α	Inhibition of viral replication	atomization: 45μg, two times daily for two weeks or 5 million units or equivalent dose, twice daily not more than 10 days
Fingolimod	Immunology modulator	0.5 mg per day orally for 3 days
Ruxolitinib	JAK1 and JAK2 inhibitor	10 mg two times a day for 14 days with dose reduction or escalation
Tocilizumab	IL-6 blocker	8 mg/kg

immunologic effect and enhancement of antibody production by diethylcarbamazine could confer its anti-COVID-19 effect. Diethylcarbamazine is expected to show the immunologic effects by the inhibition of lipoxygenase (LOX) and cyclooxygenase (COX) enzymes (Abeygunasekera and Jayasinghe, 2020). In a similar hypothesis, montelukast is suggested to be useful in restraining the progression of the disease. The anti-inflammatory effect, suppression of oxidative stress, and reduced cytokine production are supposed to facilitate the effect (Fidan and Aydoğdu, 2020). Clinical researches proved the benefit of surfactant treatment in patients with ARDS (Walmrath et al., 1996).Also it was found that early administration of natural lung surfactants can improve the pulmonary function in adult patients with severe respiratory distress syndrome (Mirastschijski et al., 2020). Surfactant based prophylactic management as well as therapy can be considered as another promising strategy (Pramod et al., 2020).

CONCLUSION

This review provides an overview of the current treatment strategies, ongoing clinical trials, and potential future options based on published research and registered clinical trials related to the COVID-19. The basics of SARS-CoV-2, the virus, and COVID-19, the disease, are provided. It was noted that none of the present therapies or strategies could be an absolute solution to end this pandemic. The drugs which have a repurposing option are selected based on its activity against RNA viruses like SARS-CoV, MERS-CoV, influenza virus, and Ebola virus. The hope of the successful vaccine, other immunological products, and cell-based therapeutics is still alive. Convalescent plasma therapy is successful in some cases. At present, it could be seen that none of the presently available approaches or drugs was able to stop this pandemic. The results of many clinical trials are yet to be announced. Further, the suggested future potential solutions seem to be promising to end this pandemic.

AUTHOR CONTRIBUTIONS

SK: conceptualization, writing – original draft, and funding acquisition. HA: supervision, writing – review and editing. SB-E: writing – review and editing. NA: supervision, writing – review and editing. SM: writing – review and editing. AN: writing – review and editing. PD: writing – review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant no. GCV19-25-1441.

ACKNOWLEDGMENTS

We therefore, acknowledge with thanks DSR for technical and financial support.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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FOI 26-3132 - Document 5

Pharmacological Reports (2021) 73:1473–1479
https://doi.org/10.1007/s43440-021-00245-z

SHORT COMMUNICATION

The association between the use of ivermectin and mortality in patients with COVID-19: a meta-analysis

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Received: 6 January 2021 / Revised: 27 February 2021 / Accepted: 6 March 2021 / Published online: 29 March 2021
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Abstract

Objective The effect of ivermectin on mortality in patients with novel coronavirus disease 2019 (COVID-19) has been investigated in many studies. We aimed to perform a meta-analysis of randomized controlled trials to investigate the overall effect of ivermectin on the risk of mortality in patients with COVID-19.

Methods We systematically searched PubMed, Cochrane Central Register of Controlled Trials, Google Scholar, and preprint repository databases (up to February 28, 2021). Random-effects and inverse variance heterogeneity meta-analysis were used to pool the odds ratio of individual trials. The risk of bias was appraised using Version 2 of the Cochrane risk-of-bias tool for randomized trials.

Results Six randomized controlled trials were included in this analysis with a total of 658 patients who were randomized to receive ivermectin and 597 patients randomized in the control group who did not receive ivermectin. Of six trials, four had an overall high risk of bias. The estimated effect of ivermectin indicated mortality benefits (pooled odds ratio = 0.21; 95% confidence interval 0.11–0.42, $n = 1255$), with some evidence against the hypothesis of 'no significant difference' at the current sample size.

Conclusion We observed a preliminary beneficial effect on mortality associated with ivermectin use in patients with COVID-19 that warrants further clinical evidence in appropriately designed large-scale randomized controlled trials.

Keywords Bias · Clinical trial · COVID-19 · Ivermectin · Mortality

Introduction

Ivermectin is well known for its efficacy against a range of parasitic diseases, including onchocerciasis and strongyloidiasis. Recently, a systematic review [1] of in vivo and in vitro studies of ivermectin demonstrated its broad antiviral activity against a range of RNA and DNA viruses. Particularly, ivermectin demonstrated an in vitro antiviral effect towards the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2]. This has since attracted the interest of frontline clinicians to investigate the potential repurposing of ivermectin for the treatment of coronavirus disease 2019 (COVID-19). Therefore, we aimed to perform a meta-analysis of randomized clinical trials to investigate the mortality benefits of ivermectin in patients with COVID-19.

Methods

We performed a systematic literature search in electronic databases including PubMed, Google Scholar, Cochrane Central Register of Controlled Trials, and preprint servers (medRxiv, Research Square, SSRN) with no language restriction, for eligible studies published up to February 28, 2021. The search strategy was built based on the following keywords and MeSH terms: "COVID-19", "SARS-CoV-2", and "ivermectin". The clinical trial registries of the United States (clinicaltrials.gov), China (chictr.org.cn), and World Health Organization (who.int/

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FOI 26-3132 - Document 5

clinical-trials-registry-platform) were also searched for registered clinical trials of ivermectin for the treatment of COVID-19 to identify studies with reported findings. Two investigators (CSK and SSH) independently performed the literature screening to identify eligible studies. The reference lists of relevant articles were also reviewed to search for additional studies. Studies eligible for inclusion were randomized controlled trials comparing the mortality outcomes of ivermectin and its comparators in patients with COVID-19. We excluded studies with observational design, single-arm trials, non-randomized trials, and trials that did not report clinical outcomes. The systematic review followed the Preferred reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [3].

The outcome of interest was all-cause mortality. Each included trial was independently evaluated by two investigators (CSK and SSH) who also extracted the study characteristics. Data collected included author(s), trial design, country, patients' age, regimen of ivermectin, regimen of comparator treatment, and mortality outcomes. Two investigators (CSK and SSH) assessed the risk of bias of the trials included with Version 2 of the Cochrane risk-of-bias tool for randomized trials (RoB 2) [4], which is a standardized method for assessing potential bias in reports of randomized interventions. RoB 2 is structured into a fixed set of domains of bias, focusing on different aspects of trial design, conduct, and reporting. We used both the random-effects model and inverse variance heterogeneity model for the meta-analysis to estimate the pooled odds ratio at 95% confidence intervals. We examined the heterogeneity between studies using the I^2 statistics and the $\chi 2$ test at 50% and $P < 0.10$, respectively. All analyses were performed using Meta XL, version 5.3 (EpiGear International, Queensland, Australia).

Results

Our systematic literature search retrieved 512 hits of which 401 were unique (titles retrieved after removing duplications) (Fig. 1). After screening, six randomized controlled trials [5–10] were included with a total of 658 patients who were randomized to receive ivermectin, and 597 patients were randomized to the control group who did not receive ivermectin. The six randomized trials that were included in the meta-analysis were respectively from Iraq [5], Iran [6], Bangladesh [7], Egypt [8], Turkey [9], and India [10]. Details of the included studies are shown in Table 1. A differing dosage regimen of ivermectin was noted across the six randomized controlled trials (Table 1).

The overall risk of bias assessed by RoB 2 is presented in Table 1. The trial by Niaee et al. [6] and Ravikirti et al. [10] had an overall low risk of bias. The other trials had an overall high risk of bias; the trial led by Mahmud et al. [7] had a high risk of bias in the domain of missing outcome data since a significant proportion (21%) of participants randomized was not included in the analysis; the trial by Elgazzar et al. [8] had a high risk of bias in the domain of randomization since the protocol for randomization was not reported; the trial by Hashim et al. [5] had a high risk of bias in the domain of randomization due to simple randomization method used with no information on allocation concealment and had also some concerns of bias in the domain of deviations from intervention due to unblinded participants; the trial led by Okumuş et al. [9] had a high risk of bias in the domain of randomization due to simple randomization method used and had also a high risk of bias in the domain of deviations from intended interventions due to open-label nature of the trial. The aforementioned four trials [5, 7–9] had a low risk of bias for other domains assessed.

The meta-analysis revealed a significantly reduced odds of mortality with the use of ivermectin among patients with COVID-19 relative to non-use of ivermectin; the estimated effect of ivermectin indicated mortality benefits (Fig. 2; pooled odds ratio = 0.21; 95% confidence interval 0.11 to 0.42, $n = 1255$) and with some evidence against the hypothesis 'no significant difference' at the current sample size.

Some may be concerned by the absence of real-time reverse transcription-polymerase chain reaction confirmatory test for COVID-19 diagnosis in a significant fraction of subjects in the trial by Niaee et al. [6], but exclusion of this trial in the meta-analysis still observed a significantly reduced risk of mortality with the use of ivermectin among patients with COVID-19 relative to non-use of ivermectin (pooled odds ratio = 0.23; 95% confidence interval 0.09–0.56).

Discussion

The mortality benefits demonstrated with the use of ivermectin in patients with COVID-19 from this meta-analysis is in agreement with the findings from the recent systematic review [1] and the largest observational study [11] on ivermectin thus far exhibiting a significantly reduced odds of mortality associated with the use of ivermectin (odds ratio = 0.27; 95% confidence interval 0.09–0.80). We, therefore, conclude a preliminary positive effect on mortality associated with ivermectin use in hospitalized patients with COVID-19 that warrants further clinical evidence in appropriately designed large-scale randomized controlled trials. Nevertheless, it should be noted that our meta-analysis was limited by a small number of patients included with a significant risk of biases in the majority of the included randomized controlled trials.

Mortality benefits elicited with the use of ivermectin in patients with COVID-19 likely stem from the ability of

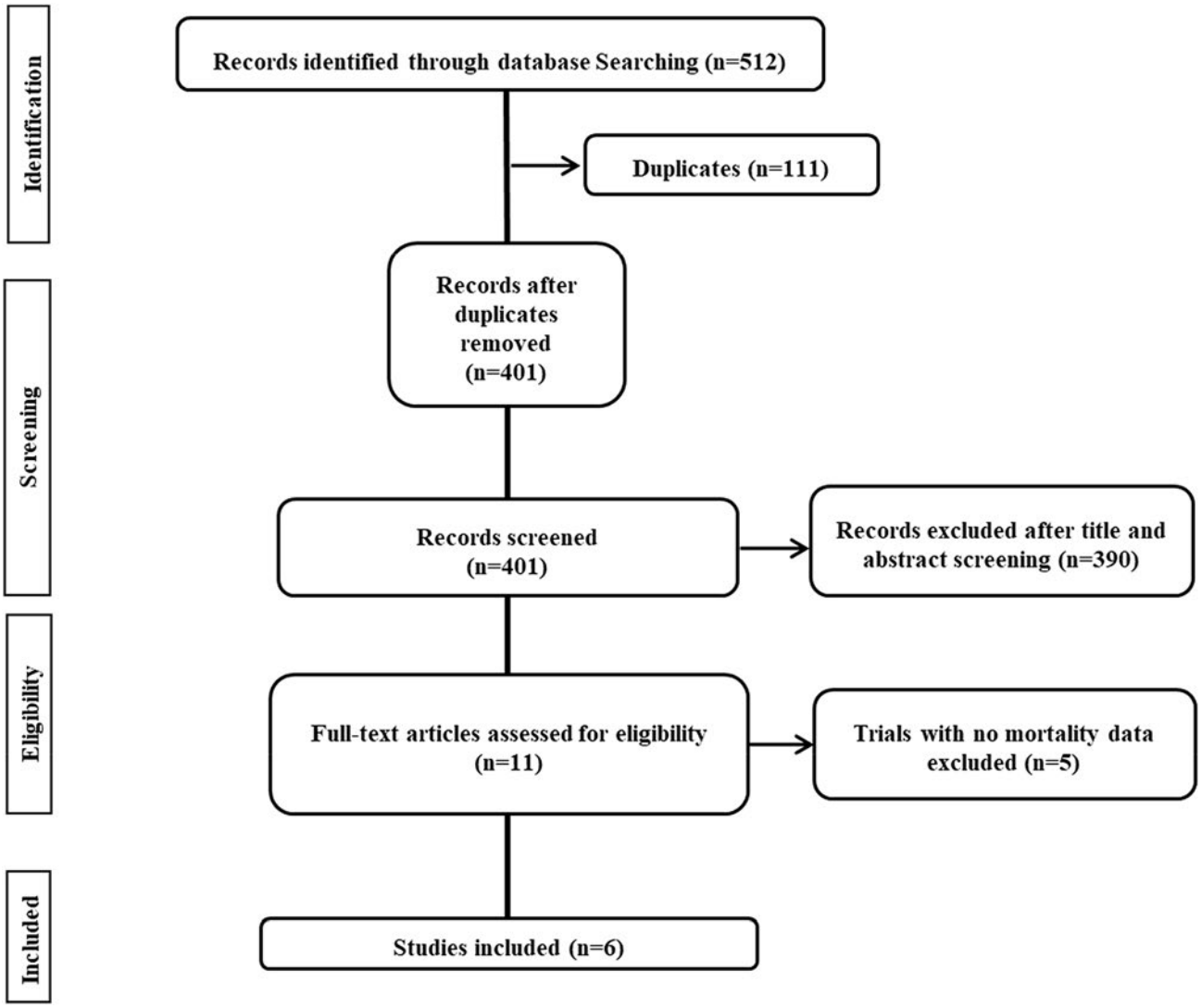


Fig. 1 PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) flow diagram of process of study selection

ivermectin to hasten clinical recovery in which a higher proportion of patients achieved clinical improvement (inconsistently defined across the included trials) in the ivermectin group compared to the control group (60.7% versus 44.4% in the trial by Mahmud et al. [7]; 96.5% versus 62.0% in the trial by Elgazzar et al. [8]; and 46.7% versus 36.7% trial by Okumuş et al. [9]). Besides, ivermectin could also prevent clinical deterioration in patients with COVID-19 wherein a lower proportion of patients deteriorated clinically (inconsistently defined across the included trials) in the ivermectin group compared to the control group (4.3% versus 10.0% in the trial by Hashim et al. [5]; 8.7% versus 17.8% in the trial by Mahmud et al. [7]; and 2.5% versus 26.0% in the trial by Elgazzar et al. [8]).

Noteworthily, the use of hydroxychloroquine in the treatment arm in the trial by Niaee et al. [6] may have confounded study outcomes. The trial by Hashim et al. [5] also offered doxycycline along with ivermectin in the intervention arm along with the standard care that also included azithromycin and corticosteroids (dexamethasone or methylprednisolone if needed). Similarly, the study by Elgazzar et al. [8] also included a complex treatment regimen where trial subjects also received azithromycin, vitamin C, zinc, lactoferrin, acetylcysteine, and prophylactic or therapeutic anticoagulation in addition to ivermectin; this was compared with a controlled group that received hydroxychloroquine. There was a potentially higher risk of cardiac arrhythmia in the control group that may have confounded the higher mortality observed (2 deaths versus 24 deaths in treatment and control groups, respectively). Indeed, RECOVERY trial had previously demonstrated a greater risk of cardiac death in COVID-19 patients on

Table 1 Characteristics of included randomized trials

Study	Study design	Country	Age (median/mean)	Regimen of ivermectin in the intervention group	Regimen of comparative intervention in the controlled group	Mortality		Risk of bias[1]
						Ivermectin users (*n*/*N*; %)	Non-ivermectin users (*n*/*N*; %)	
Hashim et al. [5]	Randomized, single-blind, controlled trial	Iraq	Ivermectin users = 50.1 Non-ivermectin users = 47.2	Oral ivermectin 200 mcg/kg per day for two days, and in patients who needed more time to recover, a third dose of 200 mcg/kg per day was given 7 days after the first dose + oral doxycycline 100 mg every 12 h for 5–10 days + standard care	Standard care (acetaminophen 500 mg if needed, vitamin C 1000 mg twice daily, zinc 75–125 mg daily, vitamin D3 5000 IU daily, azithromycin 250 mg daily for 5 days, dexamethazone 6 mg daily or methylprednisolone 40 mg twice daily, if needed)	2/70; 2.9	6/70; 8.6	High
Niaee et al. [6]	Randomized, double-blind, placebo-controlled trial	Iran	Ivermectin users = 53–61 Non-ivermectin users = 55–58	First arm: single oral dose ivermectin 200 mcg/kg Second arm: three low interval oral doses of ivermectin (200 mcg/kg) in 1, 3, and 5 interval days Third arm: single oral dose ivermectin 400 mcg/kg Forth arm: three high interval oral doses of ivermectin (400, 200, 200 mcg/kg) in 1, 3, and 5 interval days	Placebo or oral hydroxychloroquine 200 mg/kg twice daily	4/120; 3.3	11/60; 18.3	Low
Mahmud et al. (NCT04523831) [7]	Randomized, double-blind, placebo-controlled trial	Bangladesh	Ivermectin users = 40.7 Non-ivermectin users = 38.5	Oral ivermectin 1200 mcg as a single dose + oral doxycycline 100 mg every 12 h for 5 days	Placebo + standard care (acetaminophen, vitamin D, low molecular weight heparin, dexamethasone if indicated)	0/183; 0	3/180; 1.7	High

Table 1 (continued)

Study	Study design	Country	Age (median/mean)	Regimen of ivermectin in the intervention group	Regimen of comparative intervention in the controlled group	Mortality		Risk of bias[1]
						Ivermectin users (*n*/*N*; %)	Non-ivermectin users (*n*/*N*; %)	
Elgazzar et al. [8]	Randomized, double-blind controlled trial	Egypt	Ivermectin users = 56.7–58.2 Non-ivermectin users = 53.8–59.6	Oral ivermectin 400 mcg/kg (maximum 2400 mcg) once daily for four days + standard care	Oral hydroxyxholorquine 400 mg every 12 h for one day followed by 200 mg every 12 h for 5 days + standard care (azithromycin 500 mg daily for 5 days, acetaminophen 500 mg as needed, vitamin C 1 gm daily, zinc 50 mg daily, lactoferrin 100 mg twice daily & acetylcystein 200 mg three times daily, prophylactic or therapeutic anticoagulation if D-dimer > 1000)	2/200; 1.0	24/200; 12.0	High
Okumuş et al (NCT04646109)[9]	Open label, randomized controlled trial	Turkey	Ivermectin users = 58.2 Non-ivermectin users = 66.2	Oral ivermectin 200 mcg/kg/day for five days + standard care	Standard care (oral hydroxychloroquine 800 mg loading dose followed by 400 mg for 5 days, favipiravir 3200 mg loading dose followed by 1200 mg for 5 days, azithromycin 500 mg on the first day followed by 250 mg for 4 days)	6/30; 20.0	9/30; 30%	High
Ravikirti et al. [10]	Randomized, double-blind, placebo-controlled trial	India	Ivermectin users = 50.7 Non-ivermectin users = 54.2	Oral ivermectin 1200 mcg for 2 days + standard care	Placebo + standard care (hydroxychloroquine, corticosteroid, antibiotics)	0/55; 0	4/57; 7.0	Low

[1]Risk of bias was assessed using Version 2 of the Cochrane risk-of-bias tool for randomized trials

FOI 26-3132 - Document 5

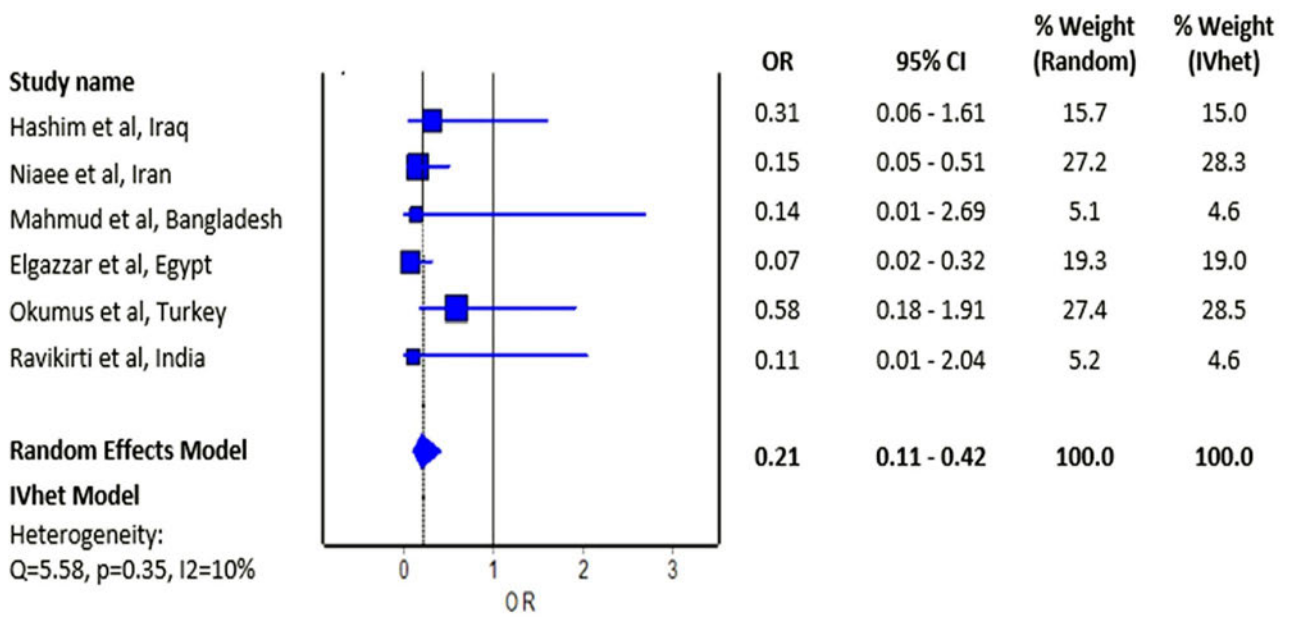


Fig. 2 Forest plot showing the pooled odds ratio of mortality between ivermectin users and non-ivermectin users with COVID-19

hydroxychloroquine [12]. On the other hand, the trial by Okumuş et al. [9] included hydroxychloroquine, favipiravir (a broad-spectrum antiviral drug), and azithromycin as part of the standard care in addition to ivermectin. It is worth mentioning that the randomized controlled trials by Niaaee et al. [6], Elgazzar et al. [8], and Okumuş et al. [9] contributed over 70% weight in this meta-analysis and therefore a more conclusive clinical evidence is still warranted to confirm the mortality benefits.

In addition, the reporting of adverse events in the included trials had been poor. Only two trials included the details on the safety of ivermectin in patients with COVID-19; the trial led by Mahmud et al. [7] reported erosive esophagitis in one patient in the treatment arm (ivermectin + doxycycline) but the use of doxycycline was likely the culprit, since doxycycline alone is one of the most common causes of drug-induced esophageal ulcers [13]; the trial led by Okumuş et al. [9] reported nausea and vomiting in two patients and increased serum levels of liver enzymes in one patient, but without serious adverse events or treatment-related adverse events with ivermectin.

We were aware of at least one treatment protocol of COVID-19 [14] at the time of writing this manuscript that recommended the use of ivermectin in patients with COVID-19. Given the reported mortality benefits with ivermectin are still preliminary, we suggest that ivermectin should preferably be administered under clinical trial settings until warranted by more conclusive large-scale randomized controlled trials.

Funding No funding or sponsorship was received for the publication of this article.

Declarations

Conflict of interest All named authors declare that they have no potential conflict of interest.

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Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

FOI 26-3132 - Document 5

Veterinary Parasitology 254 (2018) 95–97

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Wolbachia, doxycycline and macrocyclic lactones: New prospects in the treatment of canine heartworm disease

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ARTICLE INFO

Keywords:
Dirofilaria immitis
Wolbachia
Doxycycline
Macrocyclic lactones

ABSTRACT

Melarsomine dihydrochloride (Immiticide®, Merial) is the only approved adulticidal drug for the treatment of canine heartworm disease (HWD). However, in cases where arsenical therapy is not possible or is contraindicated, a monthly heartworm preventive along with doxycycline for a 4-week period, which targets the bacterial endosymbiont *Wolbachia*, might be considered. There are published reports on the efficacy of ivermectin and doxycycline in both experimentally and naturally infected dogs, but no data on the use of other macrocyclic lactones (MLs) with a similar treatment regime. Preliminary results of studies in dogs show that a topical formulation of moxidectin, the only ML currently registered as a microfilaricide, is also adulticidal when combined with doxycycline. It is not yet known if the efficacy of these combination therapies is due to pharmacokinetic synergism. A recent study showed that serum levels of doxycycline in dogs treated with the combination protocol were not statistically different compared to dogs treated with doxycycline alone. However, lungs from dogs treated with the combination therapy showed a marked reduction in T regulatory cells, indicating that treatment efficacy may be due to a heightened immune response against the parasite. Further studies are necessary to evaluate the long-term clinical outcome of combination protocols and to establish the most efficient treatment for HWD in dogs.

1. Introduction

Canine heartworm disease (HWD) is caused by the filarial nematode *Dirofilaria immitis*, a vector-borne parasite transmitted by several mosquito species. The presence of adult worms in the pulmonary arteries of infected dogs causes changes in arterial structure and function that can lead to pulmonary hypertension and, eventually, to right-sided congestive heart failure (Bowman and Atkins, 2009).

The disease is endemic in many parts of the world and currently affected areas in Europe include Spain (including the Canary Islands), Portugal, the south of France, parts of southern Switzerland, Italy, Greece, Turkey, the Czech Republic, Slovenia, Romania and Bulgaria (Genchi et al., 2014). There are several factors that may explain the spread of the parasite from endemic areas to previously unaffected areas, including climate change, density of the vector population and the movement of microfilaraemic dogs that travel throughout Europe for holidays or as “rescue” adoptions.

Melarsomine dihydrochloride (Immiticide®, Merial) is the only approved adulticidal drug for treatment of HWD. However, treatment can often be followed by severe pulmonary thrombosis (Kramer et al., 2011) and the drug is not registered for use in many countries. In cases where treatment with melarsomine is not possible or is contraindicated, a monthly heartworm preventive along with doxycycline for a 4-week period, which targets the bacterial endosymbiont *Wolbachia*, might be considered. The following review will discuss the principles behind the combination protocols and the current application of this alternative adulticide strategy in dogs with HWD.

2. *Wolbachia* is an anti-filarial target

The endosymbiont *Wolbachia* was first described in *D. immitis* by Sironi et al. (1995). Since then, *Wolbachia* has been identified in a number of filarial worm species, including several that cause human diseases like elephantiasis (*Wuchereria bancrofti*) and river blindness (*Onchocerca volvulus*) Taylor et al. (2013). Endosymbiosis has been defined as the intimate association between two different organisms maintained through generations because host and symbiont equally benefit from the association. Studies have shown that *Wolbachia* is required for normal parasite development, fertility and long-term survival of different filarial species (Taylor et al., 2013; McCall et al., 2014).

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https://doi.org/10.1016/j.vetpar.2018.03.005
Received 1 January 2018; Received in revised form 3 March 2018; Accepted 5 March 2018
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Genetic and molecular studies in the last several years have been aimed at defining exactly what these bacteria do for heartworms and other filarial nematodes (Darby et al., 2012, Godel et al., 2012). Comparison of *Wolbachia* genomes with filarial genomes have revealed that these nematodes have become dependent on their endosymbionts for a wide range of biological processes, including the production of important metabolites like heme, nucleotides and riboflavin (Darby et al., 2012; Scott et al., 2012). This was confirmed by the recent sequencing of the *D. immitis*-derived *Wolbachia* genome. Indeed, heartworm *Wolbachia* encode enzymes for anabolic pathways that are missing in the worm such as biosynthesis of heme, purine, and pyrimidines (Godel et al., 2012). Thus, current research is aimed at the removal of *Wolbachia* through antibiotic treatment of infected hosts as an adulticide strategy.

3. Doxycycline and canine HWD

Elimination of *Wolbachia* is detrimental for the filarial worm and studies on different filarial species including *O. volvulus, W. bancrofti* and *Brugia pahangi* have shown that doxycycline treatment of infected hosts causes degeneration of oocytes and early embryonic stages, is microfilaricidal and has varying adulticide effects. Death of adult worms usually takes from 18 to 24 months (Taylor et al., 2013).

Several studies have also described the effects of doxycycline in dogs with experimentally acquired HWD. Bazzocchi et al. (2008) reported that following 2–6 weeks, intermittent cycles of doxycycline treatment (10 mg/kg/day) resulted in only 8.6% efficacy against adult worms in experimentally infected dogs. However, this study evaluated worm survival at 8 months post-treatment and, as mentioned above, it is likely that death of adult worms takes longer. In a more recent study (McCall et al., 2014), dogs with experimentally-acquired patent infection were treated with doxycycline for 1 month at 20 mg/kg/day. Worm survival was evaluated at 12 and 13 months post-infection (p.i.) and results suggested that treatment had a slow-kill, adulticidal effect, which was more evident at 13, than at 12 months after doxycycline treatment started. The most interesting result of the study, however, was the inability of microfilariae from doxycycline-treated dogs that developed into infective larvae in mosquitos to successfully develop into adults after subsequent infection in new dogs. This would suggest that *Wolbachia* plays an essential role in parasite development in the mammalian host.

Indeed, doxycycline treatment has also been evaluated for its effect on developing worms. McCall et al (2011) treated experimentally infected dogs with doxycycline for 1 month at 20 mg/kg/day at different time points p.i. with third stage larvae (L3) and then verified the presence of adult worms at 8 months p.i. When doxycycline was administered during the first month p.i., no live worms were observed at necropsy, indicating 100% efficacy against infective larvae. When antibiotic treatment was given starting at 40 days p.i., efficacy against developing worms was 98.4%. Finally, when treatment was initiated 65 days p.i., efficacy against juvenile worms was 69.6%. Interestingly, none of the dogs harboring live worms were microfilariemic.

4. Macrocyclic lactone (ML)/doxycycline combination protocols

As mentioned above, doxycycline has various detrimental effects on developing and adult heartworms, but adulticide efficacy is low and slow. Macrocyclic lactones (MLs) are highly efficacious against L3 and young fourth stage larvae (L4) and prevention of disease is based on this activity. However, preventive doses of MLs have been reported as also having a so-called “slow kill” effect against adult *D. immitis* (for review see McCall, 2005). The effects of MLs on adult worms include neuromuscular dysfunction and degeneration of late-stage embryos, thus also reducing the production of microfilariae. Venco et al. (2004) evaluated the use of monthly preventive doses of ivermectin in naturally infected dogs with HWD and showed that 100% of dogs were negative for circulating microfilariae by 4 months, while 71% became negative for circulating antigens after 24 monthly doses. The study also reported a significant decrease in parasite burden as evaluated by echocardiography. However, signs of pulmonary hypertension and interstitial inflammation increased in approximately 20% of dogs and the authors concluded that this adulticide protocol should be used with caution. Furthermore, the long-term use of ML preventives in dogs with HW infection has been implicated in the development of resistant *D. immitis* strains in the southern United States (Wolstenholme et al., 2015). Thus, any method utilizing only macrocyclic lactones as a slow-kill adulticide is not currently recommended.

The hypothesis that the combination of doxycycline and MLs may have greater activity against adult heartworms than either drug alone was first tested in experimentally-infected dogs (Bazzocchi et al., 2008). Treatment with 2–6 week, intermittent cycles of doxycycline (10 mg/kg/day) combined with weekly preventive doses of ivermectin resulted in 80% adulticide activity. The adulticide effect of ivermectin/doxycycline combination protocols has also been confirmed in naturally infected dogs (Grandi et al., 2010). Importantly, the combination of ivermectin and doxycycline was well tolerated and post-adulticide complications were minimal. Indeed, it has been reported that the elimination of *Wolbachia* results in a marked decrease in pro-inflammatory responses to dead and dying worms (Kramer et al., 2008; Kramer et al., 2011; Mavropoulou et al., 2014).

It is not clear why the two drugs work better together in eliminating a large population of heart-worms in a relatively short period of time (8 – 10 months). It is not yet known if this is due to a simple summation effect or if there exists a certain synergism between the two drugs. Menozzi et al. (2015) evaluated doxycycline levels by high-performance liquid chromatography (HPLC) in sera from *D. immitis*-experimentally infected dogs treated with a combination of ivermectin/doxycycline and compared them with sera from dogs treated with doxycycline alone. Results showed that doxycycline levels were not statistically different between the two groups, suggesting that the adulticide effect is not due to a higher drug concentration of doxycycline in the combination protocol. Passeri et al. (2014) reported there was a significantly lower number of T regulatory cells in the lungs of dogs treated with a combination of both drugs when compared either to the control group or to the other groups treated with either drug alone or with melarsomine. T regulatory cells play an important role in inducing hypo-responsiveness to helminth infections (Maizels et al., 2004; D’Elia et al., 2009), so these results would suggest that successful adulticide effects of doxycycline and ivermectin are associated with a decrease in immune tolerance towards the parasite.

5. The application of ML/doxycycline combination protocols in practice

5.1. Preparation for melarsomine treatment

There have been several reports on the clinical benefits of ML/doxycycline treatment before administration of melarsomine in HW-infected dogs. Indeed, the greatest risk of adulticide therapy is the subsequent rise in pulmonary pressure due to the formation of thrombi around dead and dying worms, together with an intense inflammatory reaction against the parasite. Pre-treatment with MLs/doxy can reduce worm mass and eliminate pro-inflammatory antigens (including *Wolbachia*), thus reducing post-adulticide complications. Kramer et al. (2011) reported that experimentally-infected dogs that received a combined doxycycline/ ivermectin protocol before treatment with melarsomine showed less severe arterial lesions and the virtual absence of thrombi when compared to dogs treated with melarsomine alone. Once HWD has been diagnosed, it is currently recommended to treat dogs with 1 month of doxycycline at 10 mg/kg/BID and with 3 months of monthly preventives; at days 60, 90 and 91, treat with melarsomine with a three-step protocol. Dogs are considered cleared of infection when antigen testing is negative at 6 months post-treatment.

5.2. As an alternative adulticide

As illustrated above, ML/doxycycline combinations have been shown to be adulticidal in both experimentally and naturally-infected dogs. All published studies have concentrated on preventive doses of ivermectin, either weekly or bi-weekly for 6 months, combined with doxycycline at 10 mg/kg either SID or BID (Bazzocchi et al., 2008; Grandi et al., 2010;Mavropoulou et al., 2014). Infected dogs usually begin to be negative for circulating antigens at about 12 months from the beginning of therapy. Treatment is well-tolerated with minimal radiological and clinical signs.

A recent report has evaluated the effects of another ML in combination with doxycycline. Topical moxidectin (Advantage Multi®, Bayer) is active against infective L3-L4 larvae and against circulating microfilariae. Savadelis et al. (2016) reported in *D. immitis*-experimentally infected dogs that 9 monthly doses of topical moxidectin combined with 30 days of 10 mg/kg doxycycline BID resulted in the elimination of microfilariae by 21 days post-treatment, thus breaking the transmission cycle of the parasite very quickly. Furthermore, heartworm antigen began declining 3 months post-treatment and treatment resulted in a 95.9% efficacy in eliminating mature adult heartworms by 10 months, indicating a more marked adulticidal effect compared to that of combinations with ivermectin. It is well documented that orally administered macrocyclic lactones have a typical specific pharmacokinetic (PK) pattern showing a high peak and a very short half live and thus a very short exposure to the worm. This is dose dependent of course, but even with higher doses the general PK pattern does not change (Daurio et al., 1992). In contrast, the moxidectin PK for the spot on formulation is much different with a peak establishing several days following application, a half-life of about 28 days, and the development of a steady state level after about 4 months, ensuring a constant and high exposure of the parasite to the drug (Bowman et al., 2016).

6. Conclusions

Doxycycline and ML combinations are adulticidal for *D. immitis*. Research should, however, continue for the identification of other anti-*Wolbachia* molecules and targets. It is also necessary to further study the interaction between MLs and doxycycline/others in order to determine the best combination protocol in terms of clinical and parasitological response. Finally, as is true for any treatment of any sick dog, it is always necessary to periodically monitor the patient's response to therapy.

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JOURNAL OF VIROLOGY, Jan. 2009, p. 58–64 Vol. 83, No. 1
0022-538X/09/$08.00+0 doi:10.1128/JVI.01543-08
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Antiviral Activity of the Zinc Ionophores Pyrithione and Hinokitiol against Picornavirus Infections▽

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Received 22 July 2008/Accepted 6 October 2008

We have discovered two metal ion binding compounds, pyrithione (PT) and hinokitiol (HK), that efficiently inhibit human rhinovirus, coxsackievirus, and mengovirus multiplication. Early stages of virus infection are unaffected by these compounds. However, the cleavage of the cellular eukaryotic translation initiation factor eIF4GI by the rhinoviral 2A protease was abolished in the presence of PT and HK. We further show that these compounds inhibit picornavirus replication by interfering with proper processing of the viral polyprotein. In addition, we provide evidence that these structurally unrelated compounds lead to a rapid import of extracellular zinc ions into cells. Imported Zn^{2+} was found to be localized in punctate structures, as well as in mitochondria. The observed elevated level of zinc ions was reversible when the compounds were removed. As the antiviral activity of these compounds requires the continuous presence of the zinc ionophore PT, HK, or pyrrolidine-dithiocarbamate, the requirement for zinc ions for the antiviral activity is further substantiated. Therefore, an increase in intracellular zinc levels provides the basis for a new antipicornavirus mechanism.

Curing virus infections harbors an enormous economic potential, and the search for new antiviral substances is of great interest for worldwide health. We have previously described the commonly used NF-κB inhibitor and metal ion chelator pyrrolidine-dithiocarbamate (PDTC) to significantly inhibit the replication of several picornaviruses such as human rhinovirus (HRV), poliovirus, coxsackievirus, and mengovirus (9, 22). These examples suggest that a common step in the life cycle of these picornaviruses is the target for the antiviral drug. In particular, we have demonstrated that PDTC has negative effects on picornavirus replication by influencing the processing of the viral polyprotein (21, 22).

The antiviral activity of PDTC is not restricted to the family *Picornaviridae*, since PDTC was shown to prevent the multiplication of human influenza virus, a member of the *Orthomyxoviridae* (33, 34). However, due to strong differences in the life cycle and host-cell interaction between human influenza virus and picornaviruses, it is likely that entirely different mechanisms might be relevant for the antiviral action of PDTC against these viruses.

Currently, the precise mode of the antiviral action of PDTC is unknown, although several theories have been substantiated with experimental evidence. Antioxidative properties of PDTC are postulated to be the reason for antiviral effects against influenza virus infections (33), which is not the case for human rhinovirus multiplication (9).

We have demonstrated that the antiviral effects of PDTC are metal ion dependent, and, in particular, Zn^{2+} ions play a pivotal role. To underline the hypothesis that influx of zinc into the cells has antiviral capacity, pyrithione (PT) and hinokitiol (HK) were examined. PT is known to be a zinc ionophore that leads to a rapid increase in intracellular zinc levels (27), and HK is a chelator of divalent metal ions (2).

We provide evidence that both PT and HK inhibit replication of picornaviruses by impairing viral polyprotein processing. The basis of the antiviral activity is dependent upon the availability of zinc ions. We show that the import of extracellular zinc ions is a key feature of the common antiviral property of these compounds.

MATERIALS AND METHODS

Cell, media, and reagents. HeLa cells (strain Ohio; European Collection of Cell Cultures, Salisbury, United Kingdom) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all obtained from Gibco, Invitrogen, Austria). PDTC (pyrrolidine-dithiocarbamate ammonium salt), pyrithione (2-mercaptopyridine *N*-oxide sodium salt), Zn-EDTA, Ca-EDTA, Mg-EDTA, and *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) were purchased from Sigma-Aldrich, Austria. HK (β-thujaplicin) from Calbiochem was dissolved in dimethyl sulfoxide and stored at −20°C.

Virus preparation and titration. Strain HRV2 was obtained from the American Type Culture Collection and routinely grown in suspension cultures of HeLa cells (strain Ohio; Flow Laboratories, McLean, VA) as described previously (31). CVB3 (Nancy strain) was derived from transcription of the p53CVB3/T7 plasmid (36). The mengovirus strain EMCV was obtained upon transfection of in vitro-transcribed RNA from cDNA clone pM16.1 (5).

Virus titers in 50% tissue culture infectious doses ($TCID_{50}$) were determined according to Reed and Muench (26). From our experience, typical interassay variation is less than ±0.5 log $TCID_{50}$/ml.

Infection of cells. HeLa cells were grown in six-well plates and infected at 70% confluence with HRV2 with indicated multiplicity of infection (MOI) in RPMI medium supplemented with 2% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 2 h of infection, input virus was removed, and cells were washed three times with acidic HEPES buffer and once with phosphate-buffered saline (PBS). For further incubation, infection medium was applied for the indicated amounts of times. If indicated, chemicals were present during parts or the whole period of infection. Whenever HK was used,

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▽ Published ahead of print on 15 October 2008.

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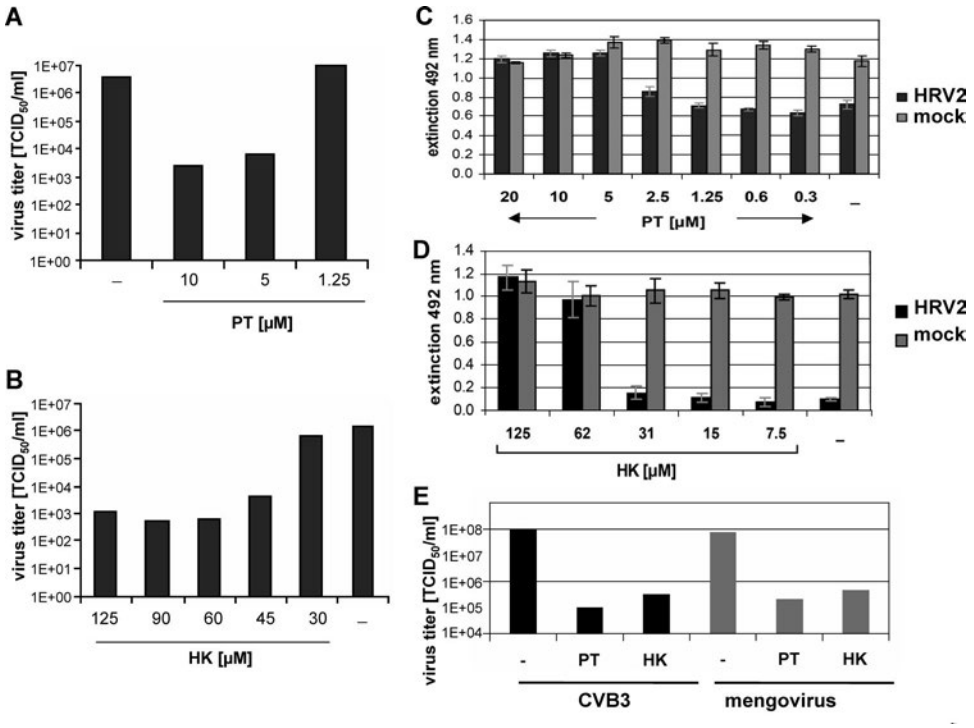


FIG. 1. PT and HK dramatically reduce picornavirus multiplication and increase cell viability. Viral titers of HRV2-infected HeLa cells (MOI = 20) in the presence or absence of indicated concentrations of PT (A) or HK plus 30 mM $MgCl_2$ (B) were determined 24 h p.i. Results for one representative experiment of three experiments are shown. HeLa cells were infected with HRV2 (MOI = 50) or mock infected, and different concentrations of PT (C) or HK-$MgCl_2$ (D) were added. At 24 h p.i., a CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay was performed according to the manufacturer's protocol. Extinction at 492 nm reflects cell viability. The means ± standard deviations of quintuples obtained in one representative experiment of three performed are shown. (E) HeLa cells were infected with CVB3 or mengovirus (MOI = 10) and incubated with 10 μM PT or 75 μM HK-30 mM $MgCl_2$, respectively. Virus production of infected cells was determined by $TCID_{50}$ assay at 24 h p.i. The results for one of two representative experiments are shown. −, control.

application of 30 mM $MgCl_2$ was essential to avoid cytotoxicity. Virus production was determined in the supernatants of infected cells by $TCID_{50}$ determination.

Confluent monolayers of HeLa cells were infected with CVB3 or mengovirus for 30 min. Cells were then washed twice with PBS and cultured in medium at 37°C in the presence or absence of indicated chemicals. At indicated time points, the cells were disrupted by three cycles of freezing and thawing, and virus titers were determined by end point titration.

Cell viability assay. HeLa cells were infected with HRV2 (MOI = 50) or mock infected in the presence or absence of different concentrations of PT or HK-$MgCl_2$. At 24 h postinfection (p.i.), a CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay (Promega, WI) was performed according to the manufacturer's protocol. Extinction at 492 nm reflecting cell viability was measured in a Labsystems Multiscan RC plate reader.

Western blot analysis. HeLa cells were infected with HRV2 as described for virus infection. At 0, 2, 4, 6, 8, and 24 h p.i., the medium was removed, and the cells were lysed by the addition of protein sample buffer. Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto polyvinylidene difluoride membranes, and analyzed using a rabbit polyclonal antibody against eIF4GI (37), as described previously (9).

Pulse-labeling and immunoprecipitation. HeLa cells were preincubated with 10 μM PT or 125 μM HK-30 mM $MgCl_2$ for 30 min and infected with HRV2 (MOI = 50) in the presence of the substances. At 6 h p.i., newly synthesized proteins were labeled with [^{32}S]Met/Cys (Hartmann Analytic, Germany) for 1 hour. At 7, 8, and 9 h p.i., VP2 or VP2-comprising precursor proteins were isolated by immunoprecipitation using the monoclonal VP2-specific 8F5 antibodies (30) and analyzed by SDS-PAGE and autoradiography as described previously (21).

HeLa cells infected with CVB3 (MOI = 50) were incubated with 125 μM PDTC, 75 μM HK-30 mM $MgCl_2$, or 10 μM PT in the presence or absence of 10 μM TPEN. Five hours p.i., cells were starved half an hour prior to 1 h of labeling with [^{35}S]Met/Cys. After labeling, cells were lysed, and cellular extracts were subjected to immunoprecipitation using either antibodies specific for 3D or 3C (kindly provided by C. Cameron, Pennsylvania State University) as described previously (22). Then, the protein pattern was analyzed by SDS-PAGE and autoradiography.

Radioactive zinc uptake studies. HeLa cells in serum-free culture medium (zinc concentration of 0.6 μM as measured by atomic absorption analysis) were incubated with 5 μM $^{65}Zn^{2+}$ (0.82 mCi/mg Zn^{2+}; Hartmann Analytic, Germany) in the presence of indicated concentrations of PDTC, PT, or HK-$MgCl_2$. At the indicated time points, the cells were harvested by filtration on GF/C glass fiber filters (Whatman, Maidstone, United Kingdom). To remove extracellular $^{65}Zn^{2+}$, filters were washed with a buffer containing EDTA (15 mM HEPES, 100 mM glucose, 150 mM KCl, 1 mM EDTA [pH 7]), and the amount of intracellular $^{65}Zn^{2+}$ was determined in a Packard Cobra II γ-counter.

Fluorescence microscopy. HeLa cells grown in μ-slide VI slides (Ibidi, Germany) were loaded with 5 μM FluoZin-3 acetoxymethyl ester (Invitrogen) or 1 μM RhodZin-3 acetoxymethyl ester (Invitrogen) in PBS for 15 min at 37°C. Subsequently, cells were incubated for 1 h in growth medium to allow deesterification. Then, PDTC, PT, or HK was added to the medium for the indicated incubation times. In some experiments, chemicals were removed after 30 min, and fresh medium was applied for 1 h. Fluorescence was monitored in a live-cell microscope system (Olympus cellR) using fluorescein isothiocyanate settings for FluoZin-3 and Cy3 settings for RhodZin-3. A Hamamatsu Photonics Orca-ER camera was used.

RESULTS AND DISCUSSION

PT and HK inhibit picornavirus multiplication and increase cell viability of infected cells. We have previously discovered that the drug PDTC substantially interferes with the multiplication of several picornaviruses (9, 22). These antiviral

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effects of PDTC are dependent on metal ions, in particular zinc ions. To further substantiate the hypothesis that zinc ions play a major role in virus inhibition, two structurally different metal ion binding compounds, PT (4, 12, 17, 35) and HK (2, 23, 32), were analyzed regarding their effects on HRV production (Fig. 1). HeLa cells were infected with HRV serotype 2 with an MOI of 20 in the presence or absence of PT or HK. At 24 h p.i., supernatants were collected, and virus titers were determined. The presence of 5 μM and 10 μM PT decreased viral titers by around 3 orders of magnitude (Fig. 1A). Likewise, HK significantly diminished viral multiplication in a dose-dependent manner (Fig. 1B). Similar results were obtained using a different HRV serotype (HRV14) or an alveolar carcinoma cell line (A549) (data not shown).

To quantify cell viability a colorimetric viability assay was performed. HeLa cells infected with HRV2 showed a significant loss of viability 24 h p.i. (Fig. 1C and D). In contrast, PT (Fig. 1C) and HK (Fig. 1D) retained viability to normal levels at concentrations between 5 μM and 20 μM PT and 62 μM and 125 μM HK, respectively. Based on additional experiments, 50% inhibitory concentrations of 3 μM for PT and 42 μM for HK were determined. Importantly, no cytotoxicity of PT and HK was found in mock-infected HeLa cells (Fig. 1C and D, gray bars) and A549 cells (data not shown) under these conditions. The absence of cytotoxicity is in contrast to reports about proapoptotic activities of PT and HK in other cell lines such as HL-60 cells (15, 19, 20).

It is of great interest to investigate whether PT and HK act specifically for HRV or whether these drugs target a common step in the picornavirus life cycle. Thus, the role of PT and HK during infection with coxsackievirus strain B3 (CVB3) and the mengovirus strain EMCV (which will hereafter be referred to as mengovirus) was examined (Fig. 1E). In a single-round infection experiment with CVB3 or mengovirus, viral titers of around 10^8 $TCID_{50}$/ml were obtained. In the presence of PT or HK, the titers were significantly reduced by around 3 and 2 orders of magnitude, respectively. These data are in agreement with the antiviral activities of PDTC against these viruses that were reported previously (22).

PT and HK abolish eIF4GI cleavage and affect viral polyprotein processing. Different steps of the HRV infection in the presence of PT or HK were studied to analyze whether PT and HK act mechanistically similar to PDTC. Early events in the viral life cycle, such as virus entry and uncoating, were not affected by PT and HK (data not shown). A hallmark of rhinovirus infection is the cleavage of the eukaryotic translation initiation factors eIF4GI and eIF4GII by the viral $2A^{pro}$ (7, 11), leading to the shutoff of host-cell protein synthesis (8). To test whether PT and HK affect eIF4GI cleavage, protein extracts of HRV2-infected cells were analyzed by Western blotting (Fig. 2A). In the absence of PT or HK, around 50% cleavage of eIF4GI was obtained at 4 h p.i. At 6 h and 8 h p.i., eIF4GI was nearly completely processed into its typical cleavage products. In the presence of PT or HK, no significant amounts of eIF4GI cleavage products were found within 24 h of infection. This is in agreement with data obtained with PDTC (9).

As PDTC shows a clear inhibition of viral polyprotein processing (21), P1 processing was also examined in the

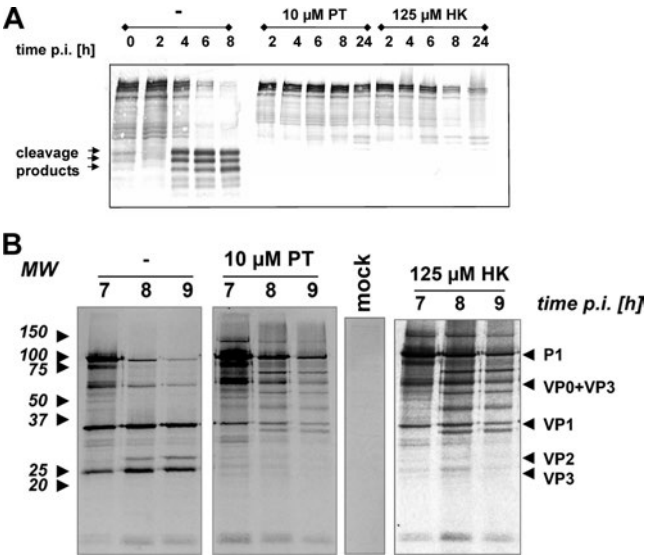


FIG. 2. PT and HK abolish HRV2-triggered eIF4GI cleavage and affect viral polyprotein processing. (A) HRV2-infected HeLa cells (MOI = 20) were either mock treated or treated with 10 μM PT or 125 μM HK-30 mM $MgCl_2$. After the indicated time points, protein extracts were collected and analyzed by Western blotting using an antibody specific for eIF4GI and its cleavage products. (B) HeLa cells were preincubated with 10 μM PT or 125 μM HK-30 mM $MgCl_2$ for 30 min and infected with HRV2 (MOI = 50) in the presence of the substances. At 6 h p.i., newly synthesized proteins were labeled with [^{32}S]Met/Cys for 1 hour. At the indicated time points, VP2 or VP2-containing precursor proteins were isolated by immunoprecipitation and analyzed by SDS-PAGE and autoradiography. Immunoprecipitation of mock-infected labeled cells did not show any signal. MW, molecular weight in thousands.

presence of PT or HK (Fig. 2B) by a pulse-chase labeling experiment, followed by immunoprecipitation with a VP2-specific antibody, 8F5, which pulls down VP2, VP2-containing precursors, and viral proteins bound to VP2 (30). Labeling of viral proteins started at 6 h p.i. for 60 min, and subsequently, cell lysates were prepared every hour. In untreated cells, the processing of the P1 precursor protein was clearly visible between 7 h and 9 h p.i. as a reduction of the amount of the corresponding polyprotein band. At the same time, smaller bands comprising VP2 and VP3 appeared. In the presence of either PT or HK, the processing of the P1 protein was blocked, and no mature VP2 or VP3 products could be detected. Moreover, various intermediate processing products were obtained in the presence of PT or HK, giving an identical picture to the processing defects observed using PDTC (21).

To specifically monitor the activity of $3CD^{pro}$, which is responsible for the processing of the P1 region and which autocleaves itself into $3C^{pro}$ and $3D^{pol}$, the polyprotein-processing pattern of coxsackievirus infection in the presence of PDTC, PT, and HK was investigated (Fig. 3). CVB3-infected HeLa cells incubated with PDTC, HK, or PT were starved half an hour prior to 1 h of labeling at 5.5 h p.i. Subsequently, immunoprecipitation experiments using polyclonal antibodies raised against CVB3 $3C^{pro}$ and $3D^{pol}$ were performed and analyzed as described above. PDTC, PT, and HK abolished coxsackievirus polyprotein processing into the

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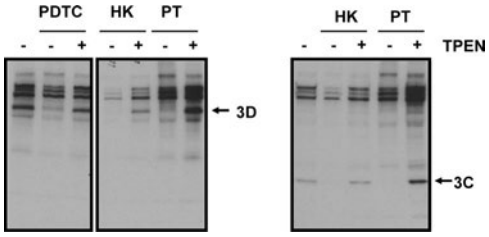


FIG. 3. PT and HK abrogate 3D and 3C processing of CVB3. HeLa cells infected with coxsackievirus B3 (MOI = 50) were incubated with 125 μM PDTC, 75 μM HK-30 mM $MgCl_2$, or 10 μM PT in the presence or absence of 10 μM TPEN. At 5 h p.i., cells were starved half an hour prior to 1 h of labeling with [^{35}S]Met/Cys. Harvested cell lysates were subjected to immunoprecipitation using either antibodies specific for 3D (left panel) or 3C (right panel) and then analyzed by SDS-PAGE and autoradiography. Results for a representative experiment are shown. −, TPEN absent; +, TPEN present.

mature viral proteins 3D and 3C in a similar manner indicating a common antiviral mechanism of all three compounds. The presence of 10 μM of the strong metal ion chelator TPEN restored viral polyprotein processing, suggesting that the observed inhibition is based on the availability of metal ions.

These data indicate that polyprotein processing of picornaviruses is a sensitive step for treatment with zinc ionophores. We have evidence that viruses such as respiratory syncytial virus (belonging to the family of *Paramyxoviridae*) which do not depend on polyprotein processing during their life cycle are not inhibited by PDTC treatment (data not shown). An unspecific adverse effect of PDTC on general cellular functions that lead to virus inhibition is therefore unlikely.

EDTA abolishes the antipicornavirus effects of PT and HK. We and others have shown that treatment with PDTC or PT leads to a rapid elevation of the concentration of free cytoplasmic Zn^{2+} ions (6, 22, 16–18). The importance of metal ions was further substantiated by experiments using EDTA as a general extracellular metal ion chelator (Fig. 4). In the presence of 10 μM EDTA, the antiviral properties of PT and HK against HRV2 (Fig. 4A), CVB3 (Fig. 4B), and mengovirus (Fig. 4C) are lost, indicating the involvement of metal ions. By using Zn^{2+}-, Ca^{2+}-, or Mg^{2+}-saturated EDTA (25), we demonstrate that only Zn-EDTA retains the antiviral property of PT or HK. This indicates that the availability of Zn^{2+} ions is a prerequisite for the antiviral activity of these compounds. None of the EDTA-metal ion complexes alone had any antiviral effect at the concentrations tested.

PDTC, PT, and HK treatments cause a rapid and efficient influx of $^{65}Zn^{2+}$ into cells. It is unclear whether PDTC, PT, and HK mobilize zinc ions localized in intracellular storage vesicles or allow extracellular ions to enter the cytoplasm. To directly monitor import of extracellular zinc ions into HeLa cells, we employed radioactive $^{65}Zn^{2+}$ to determine the effects of PT, HK, and PDTC on zinc import (Fig. 5A). HeLa cells were incubated for different time periods with 5 μM $^{65}Zn^{2+}$ and PDTC, PT, or HK and then harvested by filtration on GF/C glass fiber filters. The amount of intracellular $^{65}Zn^{2+}$ was measured by γ-counting of the filter membranes. In the presence of $^{65}Zn^{2+}$ alone, no significant increase in radioac-

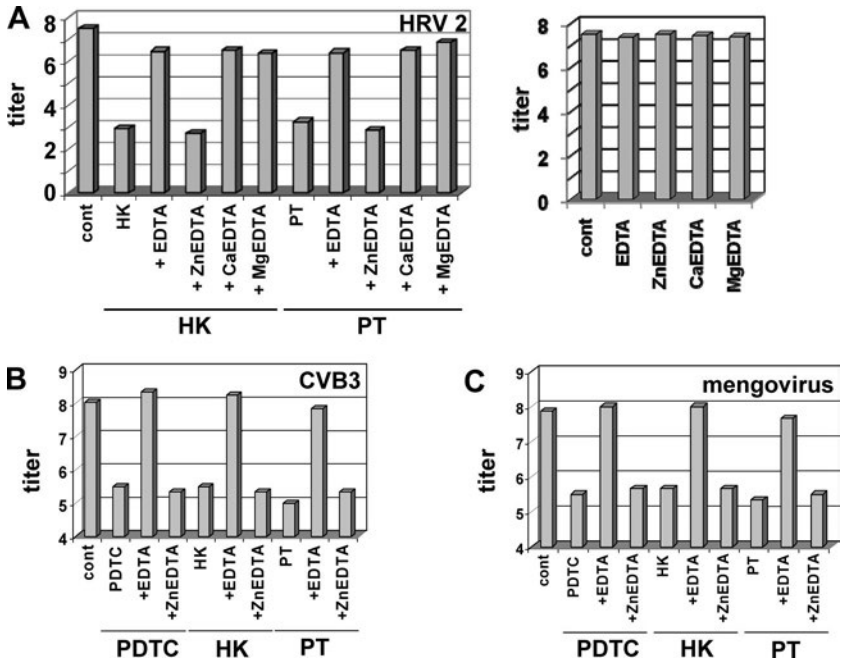


FIG. 4. Zinc ions are responsible for the antiviral property of PT and HK. HeLa cells were infected with HRV2 (MOI = 20) (A), CVB3 (MOI = 10) (B), or mengovirus (MOI = 10) (C) and incubated with 10 μM EDTA or 10 μM of Zn-EDTA, Ca-EDTA, and Mg-EDTA, respectively. If indicated, 10 μM PT (A, B, and C), 125 μM HK-30 mM $MgCl_2$ (A), or 75 μM HK-$MgCl_2$ (B and C) was added at the time of infection. The virus titers in the supernatants of the infected cells were determined by a $TCID_{50}$ assay at 24 h p.i. and shown as log $TCID_{50}$/ml. Viral infection without addition of compounds is labeled as "cont." The results for one representative experiment of three are shown.

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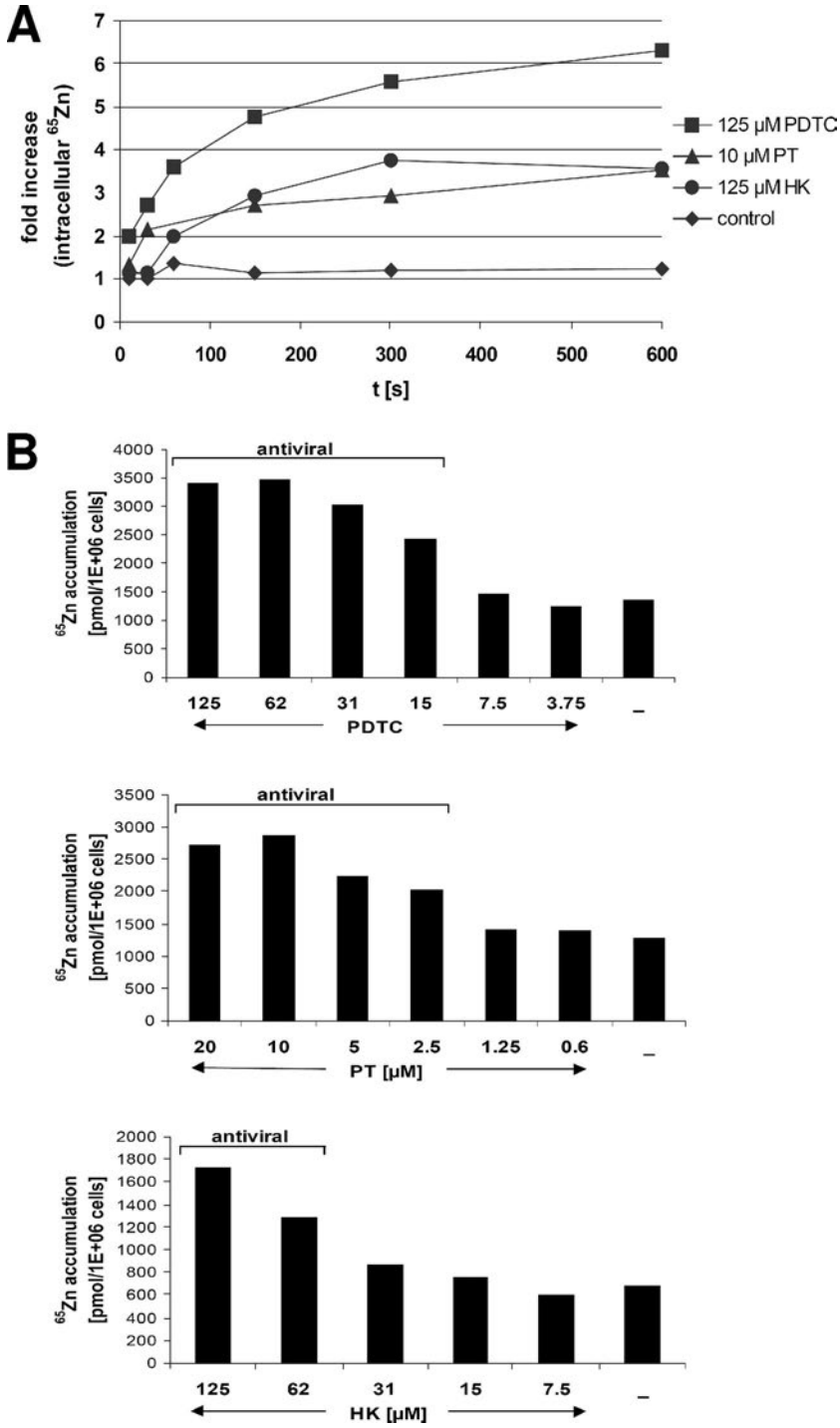


FIG. 5. PDTC, PT, and HK cause import of extracellular $^{65}Zn^{2+}$ levels in a dose-dependent manner. (A) HeLa cells in serum-free culture medium were incubated with 5 μM $^{65}Zn^{2+}$ and 125 μM PDTC, 10 μM PT, or 125 μM HK-30 mM $MgCl_2$. At the indicated time points, the cells were harvested and washed with a buffer containing EDTA, and the amount of intracellular $^{65}Zn^{2+}$ was determined in a Packard Cobra II γ-counter. Statistical variations between different samples are determined to be a maximal ±10% (data not shown). The results for one of three experiments are shown. (B) HeLa cells in serum-free culture medium were incubated with 5 μM $^{65}Zn^{2+}$ and various concentrations of PDTC (top panel), PT (middle panel), or HK-$MgCl_2$ (lower panel) for 15 min. Then, the cells were collected and washed, and the amount of intracellular $^{65}Zn^{2+}$ was determined as described above. The results for one of five experiments are shown.

tivity of the collected cells was measured, whereas the presence of either PT or HK facilitated a threefold increase of intracellular $^{65}Zn^{2+}$ within a few minutes. PDTC elevated the intracellular $^{65}Zn^{2+}$ level sixfold. These results demonstrate that all three substances facilitate import of extracellular zinc ions via a rapid uptake mechanism.

As shown in Fig. 5B, this zinc uptake is dose dependent. The presence of more than 15 μM PDTC, 2.5 μM PT, or 62 μM HK

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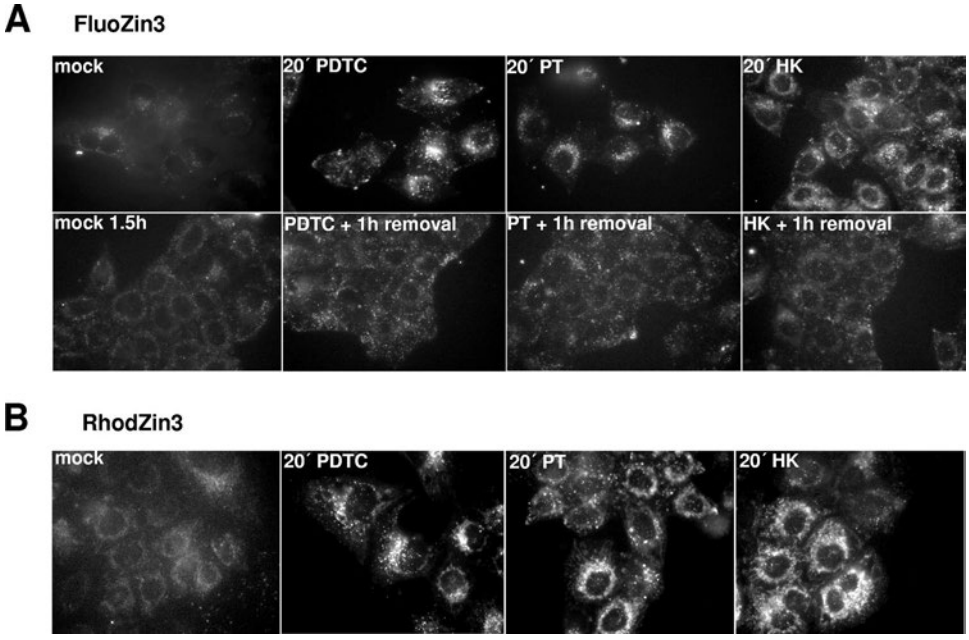


FIG. 6. Zinc ionophores influence the labile Zn^{2+} pool. HeLa cells were loaded with 5 μM FluoZin-3 (A) or 1 μM RhodZin-3 (B) in PBS for 15 min at 37°C. After extensive washing and 1 h of de-esterification in medium, zinc uptake was induced by treatment with 125 μM PDTC, 10 μM PT, and 125 μM HK-30 mM $MgCl_2$ in growth medium. If indicated, after 30 min of chemical treatment, ionophores were removed for 1 hour (PDTC + 1 h removal, PT + 1 h removal, HK + 1 h removal). Pictures were taken at the indicated time points by live fluorescence microscopy with 60× optics. Examples of images of three independent experiments were chosen.

caused significant $^{65}Zn^{2+}$ uptake after 15 min. It is noteworthy that this concentration range was found to be antiviral (Fig. 1) (9), suggesting that the influx of zinc ions is essential for mediating the common antiviral property of these compounds.

Intracellular localization of zinc ions imported via PT, HK, and PDTC. To directly visualize the ability of PDTC, PT, and HK to increase the intracellular pool of labile Zn^{2+}, specific zinc indicators were used (Fig. 6). FluoZin-3 is a Zn^{2+}-sensitive and -specific fluorescent probe that has no specific cellular localization (14). In cells loaded with FluoZin-3, intracellular fluorescence was minimal (Fig. 6A, top panel) due to a very low level of free zinc ions (24). After induction with 10 μM PT, 125 μM PDTC, or 125 μM HK, a rapid and significant increase in fluorescence was found reflecting an elevation of the intracellular labile "chelatable" zinc level. Noteworthy, these experiments were carried out in normal growth medium and apparently the Zn^{2+} concentration present in the medium is sufficient to lead to ionophore-mediated zinc ion transport.

The observed punctate pattern of FluoZin-3 fluorescence is in agreement with recent data from rainbow trout cells incubated with high levels of extracellular zinc (24). In a large variety of mammalian cell types, vesicular storage sites for zinc which have been designated "zincosomes" can be detected (1). Zincosomes play a role in detoxifying excess zinc and are suggested to colocalize with endosomal compartments and lysosomes (13).

The uptake of zinc ions is reversible. When the zinc ionophores PDTC, PT, and HK were removed, the fluorescence declined close to background within 1 h (Fig. 6A, bottom panel).

To specifically monitor the free mitochondrial zinc pool, RhodZin-3 was used (28). HeLa cells loaded with RhodZin-3 exhibited a very weak fluorescence, indicating low levels of labile Zn^{2+} inside the mitochondrial compartment (Fig. 6B). In contrast, upon 20 min of treatment with PDTC, PT, or HK, the signal of RhodZin-3 fluorescence was strongly enhanced. This mitochondrial localized signal was found to be constant over at least 80 min of PT incubation (data not shown). The role of a labile Zn^{2+} fraction in mitochondria during viral infection is currently unclear and has to be the subject of further studies.

Concluding remarks. Conclusively, all three antiviral substances are potent zinc ionophores increasing the intracellular pool of labile zinc. This strong elevation is constant as long as the zinc ionophores are present but is reversible after removal of PDTC, PT, or HK.

The reversibility of the elevation of free-zinc concentrations is in agreement with data that we had obtained when virus multiplication was monitored in a setup when PT, HK, and PDTC were present for short intervals only. In contrast to the significant viral titer reduction evoked by permanently present PDTC, PT, or HK, a short (1 h) pulse of zinc ionophore treatment between 2 h and 5 h p.i. was not sufficient to significantly reduce viral titers (data not shown). These data indicate that a prolonged presence of ionophores during viral replication is needed to exert antiviral effects. In previous data, we have shown that a clear reduction of virus titer was obtained when PDTC treatment started up to 4 h after infection, continuing to 24 h (9).

In conclusion, we have identified a set of three antiviral compounds against HRV, coxsackievirus, and mengovirus infections, which differ in their chemical structures but appear to use import of zinc ions as a common trigger for exerting their antiviral effect. It was reported that PDTC reduces coxsackie-

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virus B3 replication through inhibition of the ubiquitin-proteasome pathway (29). We tested the proteasome inhibitor MG-132 at nontoxic concentrations for antiviral properties against HRV2 and HRV14. However, functional MG-132 concentrations that cause a massive accumulation of ubiquitinylated proteins within 8 h of incubation were not antiviral (data not shown). Thus, proteasomal inhibition is not likely to be the major cause of the antiviral activity of PDTC, PT, or HK.

Importantly, PDTC, PT, and HK lead to efficient import of zinc ions that are naturally present in the cell culture medium. Uptake of Zn^{2+} into cells interferes with the viral life cycle. Already in 1974, Butterworth and colleagues have shown that adding high concentrations of zinc ions to cells impairs picornavirus polyprotein processing (3); however, the mechanistic basis still remains incompletely understood. On the one hand, viral proteases might be affected in their functions directly; on the other hand, zinc ions could contribute to folding problems of the viral polyprotein that lead to inseparable precursors. Folding inhibition was recently suggested as a target for antiviral strategies (10).

Several attempts have been employed to create mutants resistant against PDTC during human rhinovirus or influenza virus infections. However, no resistant viruses were obtained, hinting at a cellular mechanism of action which is expected to be similar for HK and PT. On account of these findings, PDTC, PT, and HK may provide an interesting basis for the development of new classes of antipicornaviral therapeutics.

ACKNOWLEDGMENTS

This work was supported by grant LS05-039 of the Vienna Science and Technology Fund to J.S. and by grant NWO-VIDI-6 917.46.305 of The Netherlands Organization for Scientific Research and the M.W. Beijerink Virology Fund from the Royal Netherlands Academy of Sciences to F.J.M.V.K.

We thank C. Cameron (Pennsylvania State University) for the kind gift of antibodies, Andrea Trienoll for technical help, and Hans Goldenberg for helpful discussions.

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Antiviral effect of high-dose ivermectin in adults with COVID-19: a pilot randomised, controlled, open label, multicentre trial.

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Summary

Background

There are limited antiviral options for the treatment of patients with coronavirus disease 2019 (COVID-19) that have demonstrated clinical efficacy and none of them is an oral drug. Ivermectin (IVM), a macrocytic lactone with a wide anti-parasitary spectrum, has shown potent in vitro activity against SARS-CoV-2 in cell cultures.

Methods

We completed a pilot, randomized, controlled, outcome-assessor blinded clinical trial with the goal of evaluating the antiviral activity of high dose IVM in COVID-19 patients. Eligible patients were adults (aged 18 to 69 years) with mild or moderate RT-PCR confirmed SARS-CoV-2 infection within 5 days of symptoms onset. 45 patients were randomized in a 2:1 ratio to standard of care plus oral IVM at 0·6 mg/kg/day for 5 days versus standard of care. The primary endpoint was viral load reduction in respiratory secretions at day-5. Viral load in respiratory secretions was measured through quantitative RT-PCR. Concentrations of IVM in plasma were measured on multiple treatment days. This trial is registered with ClinicalTrials.gov, NCT004381884.

Findings

The trial run between May 18 and September 29, 2020 with 45 randomized patients (30 in the IVM group and 15 controls). There was no difference in viral load reduction between groups but a significant difference in reduction was found in patients with higher median plasma IVM levels (72% IQR 59 – 77) versus untreated controls (42% IQR 31 – 73) (p=0·004). The mean ivermectin plasma concentration levels also showed a positive correlation with viral decay rate (r:0·47, p=0·02). Adverse events were reported in 5 (33%) patients in the controls and 13 (43%) in the IVM treated group, without a relationship between IVM plasma levels and adverse events.

Interpretation

A concentration dependent antiviral activity of oral high dose IVM was identified in this pilot trial at a dosing regimen that was well tolerated. Large trials with clinical endpoints are necessary to determine the clinical utility of IVM in COVID-19.

Funding: This work was supported by grant IP-COVID-19-625 from Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación, Argentina and Laboratorio ELEA/Phoenix, Argentina.

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Research in context

Evidence before this study

The potential role of ivermectin against SARS-CoV-2 was first reported in April 2020 when an Australian group published in-vitro results. Since then, multiple opinion papers and uncontrolled studies tried to understand the meaning of those results and utility of ivermectin in COVID-19. We searched clinicaltrials.gov on October 16 and identified 39 registered trials. A Pubmed search on the same date identified 72 published articles on ("COVID" or "SARS") AND "ivermectin", none of them a clinical trial o randomized controlled trial.

Added value of this study

Our study is the first contribution that provides evidence of the antiviral activity of ivermectin against SARS-CoV-2 in patients with COVID-19 through a randomised, controlled, outcome-assessor blinded clinical trial. The incorporation of quantitative viral load determinations and measurements of ivermectin plasma levels allows an in-depth interpretation of the data and the identification of ivermectin systemic concentrations needed for a significant antiviral effect. The use of an untreated control group highlights the need for controlled trials and on the viral load dynamics in the natural history of COVID-19. Finally, we also add further information on the safety of high dose ivermectin.

Implications of all the available evidence

A concentration dependent antiviral effect of ivermectin in COVID-19 was identified, with significant reductions in SARS-CoV-2 viral load in respiratory secretions among patients achieving high systemic ivermectin concentration compared to untreated controls. These results, that did not show toxicity related to the use of high dose ivermectin, provide evidence of the antiviral effect and support the design of trials to investigate the clinical implications of our findings. Further exploration of the factors involved in the oral bioavailability of ivermectin are also warranted.

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Introduction

The emergence of a novel coronavirus, Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) in Wuhan in December 2019 and its pandemic spread causing (COVID-19) at a global scale, with over 33 million reported cases and 1 million deaths by the end of September 2020 has prompted the search of pharmacologic interventions to treat, prevent and mitigate the consequences of this potentially devastating acute respiratory infection. Several therapeutic agents have been evaluated at different disease stages as potential antiviral therapies; most of them as part of a drug repurposing strategy for active principles already used in other therapeutic indications. Although different molecules such as hydroxychloroquine, lopinavir and remdesivir have demonstrated antiviral activity against SARS-CoV-2 *in vitro*, evidence from randomized controlled clinical trials has only demonstrated clinical benefits for intravenous remdesivir in sub-groups of hospitalized patients. [1]

Ivermectin (IVM) is a widely used antiparasitic drug against several filarial diseases, scabies, and strongyloidiasis, with over 900 million tablets distributed in 2019 through the World Health Organization (WHO) for the treatment of onchocerciasis and lymphatic filariasis as part of WHO`s Essential Medicines List. [2,3] After decades of intensive, safe, and effective use, IVM has more recently been evaluated for potential new indications including malaria and several viral infections that were shown to be susceptible to IVM in vitro like Dengue, Zika, and Influenza. [4] A potent activity against SARS-CoV-2 was reported in Vero-hSLAM cells cultures using high concentrations of IVM.[5] Different pharmacokinetic (PK) and pharmacodynamic (PD) models suggested that the extremely high plasma concentrations necessary for the antiviral effect observed *in vitro* would require doses far from those safely used in humans, in order to reach concentrations close to 2 μM, the half-maximal inhibitory concentration (IC50) required against SARS-CoV-2 *in vitro.* [6] IVM is prescribed in weight-based regimens, most frequently at 0·2 mg/kg, although 0·4 mg/kg is approved for *Wuchereria bancrofti* infections.[7] Based on its safety profile, higher dosing regimens are under evaluation due to their potential utility for new indications and dosing strategies. [7,8]

To evaluate the antiviral activity and safety profile of high dose IVM in COVID-19 patients and to advance the knowledge on the role of this drug, we completed a proof of concept randomized controlled clinical trial in hospitalized patients with mild and moderate disease. To achieve further insights into the potential clinical utility of IVM in COVID-19, the relationship between drug PK (systemic exposure) and PD (dynamic of the viral load) aspects was investigated. Here we present the results of the trial with descriptions on the impact of IVM on SARS-CoV-2 viral load in respiratory secretions.

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Methods

Study design and participants

A multicenter, individually randomized, open label, outcome assessor blinded, controlled clinical trial to assess the antiviral activity and safety of a 5-day regimen of high dose IVM versus no treatment in a 2:1 allocation ratio, in adult hospitalized patients with mild to moderate COVID-19. All patients in both groups received standard of care. The trial was done at 4 hospitals in the metropolitan area of Buenos Aires, Argentina.

Ethical approval was obtained from the Institutional Independent Ethics Committees and from district and national regulatory agencies. All participating individuals provided written informed consent. The trial was done in accordance with the principles of the Declaration of Helsinki. This study is registered with ClinicalTrials.gov, NCT004381884.

Eligible participants were COVID-19 patients aged 18 to 69 years-old with RT-PCR confirmed infection, hospitalized with disease stages 3 to 5 from the WHO 8-Category ordinal scale of clinical status and no requiring intensive care unit admission. Eligibility criteria included COVID-19 symptoms onset $\leq$ 5 days at recruitment, absence of use of drugs with potential activity against SARS-CoV-2 and available in Argentina during the trial (hydroxychloroquine, chloroquine, lopinavir and azithromycin); and those drugs were not permitted during the first week of the trial. Exclusion criteria included the use of immunomodulators within 30 days of recruitment, pregnancy, breast feeding, poorly controlled comorbidities and known allergies to IVM. Patients of child-bearing age (men and women) were eligible if agreed to take effective contraceptive measures (including hormonal contraception, barrier methods, or abstinence) during the study period and for at least 30 days after the last study drug administration.

Randomization and masking

Enrolled participants were randomly assigned (2:1) to either IVM group or untreated control group. Randomization was stratified for each Center. Randomization sequence was prepared by a centralized, web-based system in blocks of variable size (3, 6 or 9 cases per block) and communicated to the trial physicians that recruited the patients upon entry to the web system information on availability of the signed Informed Consent Form and verification of all eligibility criteria. Patients, nurses, and physicians were not blinded to the treatment arm. Outcome assessors (virology staff) were blinded to the treatment group by receiving the samples labeled with randomization code and visit number.

Procedures

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All participating patients were evaluated at study entry when a full history and physical exam were performed, including weight and height. Patients in the IVM group received oral treatment for 5 consecutive days with either breakfast or lunch at approximately 24 hours intervals. IVM 6 mg ranurated tablets (IVER P, Laboratorios Elea/Phoenix, Argentina) were used in all cases at a dose of 0·6mg/kg/day based on baseline weight rounding to the lower full (6mg) and half (3mg) dose. Patients were assessed daily by hospital nurses and physicians from the research teams in each hospital during the 7-day initial period and at day 21 to 30 from study entry. All clinical findings, including adverse events and concomitant medication, were entered in paper based clinical records and then entered into an electronic case report form that was monitored and validated by trial staff. Nasopharyngeal swabs were collected at baseline and 24, 48 and 72 hours and on day 5 for SARS-CoV-2 viral load quantification. Blood samples were obtained by venipuncture for plasma IVM concentrations, 4 hours after drug intake on treatment days 1, 2, 3, and 5 (aiming at measuring peak plasma levels) and on day 7 (aiming to estimate drug elimination) in the IVM group. Blood samples were obtained from participants in both groups for hematologic and chemical parameters.

Outcomes

The primary outcome measure was the reduction in SARS-CoV-2 viral load between baseline and day-5 in both groups. Secondary outcomes included clinical evolution at day-7, relationship between IVM plasma concentrations and the primary outcome, and frequency and severity of adverse events in each group.

SARS-CoV-2 viral load measurements

Viral load of SARS-CoV-2 from nasopharyngeal swabs was quantified from samples stored in viral transport medium at -80ºC until use. For the viral load assay, viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from 140 µL of stored samples. Then, an in-house reverse transcriptase quantitative PCR (RT-qPCR) targeting N gene of SARS-CoV-2 was performed. The standard curve consisted of an in vitro transcribed viral RNA serially diluted in a cellular RNA matrix from negative nasopharyngeal samples. This assay included the measure of a housekeeping gene as an internal control and normalizer. The housekeeping gene cycle threshold (Ct) was used to correct the specific-SARS-CoV-2 Ct according to the number of cells in the sample. Therefore, viral load measurements were expressed as $\log_{10}$ copies per reaction instead of $\log_{10}$ copies per mL as discussed by Han and colleagues. [9] The performance of the assay includes: i) efficiency = 99%, ii) reproducibility with a coefficient of variation (CV) between 1·01 to 2·31, iii) repeatability with a CV between 0·27 to 1·89%, iv) dynamic range from 10 to 1 x 10^8 copy per reaction, v) specificity = 100% tested against SARS-CoV-2 negative samples and a panel of respiratory viruses. All these parameters were determined according to the guidelines for in vitro quantitative diagnostic assays as were reported previously. [10,11]

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Measurement of IVM plasma concentration profiles

Concentrations of IVM in plasma samples collected from treated patients were determined by High-Performance Liquid Chromatography (HPLC) with fluorescence detection. The chromatography technique was adapted as previously described. [12] An aliquot of plasma was combined with moxidectin (used as internal standard). After an acetonitrile-mediated chemical extraction, IVM was converted into a fluorescent molecule using N-methylimidazole and trifluoroacetic anhydride (Sigma Chemical, St Louis, MO, USA). An aliquot (100 µL) of this solution was injected directly into the HPLC system (Shimadzu Corporation, Kyoto, Japan). HPLC analysis was undertaken using a reverse phase C18 column (Kromasil, Eka Chemicals, Bohus, Sweden, 5 µm, 4.6 mm × 250 mm) and an acetic acid 0·2% in water/methanol/acetonitrile (1·6/60/38·4) mobile phase at a flow rate of 1·5 mL/min at 30 °C. Fluorescent detector was set at 365 nm (excitation) and 475 nm (emission wavelength). Full validation of the analytical procedures used to measure IVM plasma concentrations was performed. The determination coefficient (r^2) of the calibration curve was 0·995. The mean absolute drug recovery percentage was 94%. The precision of the method showed a coefficient of variation below 8·1%. The limit of drug quantitation was 0·3 ng/mL. Drug concentrations in experimental plasma samples were obtained by peak area integration using the Solution Software (Shimadzu Corporation, Kyoto, Japan).

Pharmacokinetic and pharmacodynamic analysis of the data

The drug plasma concentrations measured in each patient at the different post-treatment times were plotted. The pharmacokinetic parameters were determined using PK Solutions 2·0 (Ashland, Ohio, US) computer software. The area under the IVM concentration-time curves for (AUCivm) (named as systemic drug exposure) were calculated by the trapezoidal rule.[13] The estimation of the area under the viral load-time curves (AUCvl) was estimated as a pharmacodynamic indicator of drug activity. The viral decay rate was calculated from the viral load curve-time using 4 points as 2·303 x slope.[13] The data analysis included the estimation of the drug PK/PD relationship. The ratio between drug exposure (PK) (expressed as AUCivm) and the dynamic of the viral load (PD) (expressed as AUCvl) was estimated as an indicator of the relationship between drug exposure and antiviral activity.

Statistical analysis

Trial design included a sample size calculation that was determined on current recommendations for pilot trials, indicating at least 10 cases per group [14]or based on the sample size calculation for

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the full-scale clinical trial and include at least 9% of that size for a confidence interval of 80% (Cocks).[15] Based on these grounds and low effect size (0·3) the sample size for a full-scale trial would be 342 and a pilot of at least 31 based on Cocks *et al*. In view of the presumed effect of IVM on the replication of SARS-CoV-2 and the limited available information of viral dynamics at the time of study design (April 2020), the sample size of the pilot trial with an α error of 0·05 a power of 0·80 and a low effect size according to standardized size effects,[16] was calculated for a 2:1 randomization in 30 patients in the IVM group and 15 in the control group, to detect differences between 2 independent groups in the decrease in mean viral load in nasopharyngeal swabs between baseline and day-5.

Groups were compared and the continuous variables were analyzed for statistical significance with the Mann-Whitney and the Kruskal-Wallis non-parametric test. Correlations were analyzed using the Spearman rank test. Data analysis was performed with GraphPad Prism version 5·00 for Windows (La Jolla California USA (www.graphpad.com)). The statistical analysis for PK parameters and percentages of viral load reduction at day 5 post-treatment was performed using the Instat 3·0 software (GraphPad Software, CA, US). Parametric (ANOVA, Bonferroni multiple comparisons) and non-parametric (Kruskal–Wallis) tests were used for the statistical comparison. When difference across three groups was significant, pairwise comparisons were made with Dunn`s multiple comparisons test. Non-parametric correlation was used for the relationship between viral load reduction and IVM systemic concentration. In all cases, p-values <0·05 were considered statistically significant. Two missed values of viral load were estimated by regression analysis using the existing data.

Role of the funding source

The sponsors of the study participated in study design, but had no role in primary data collection, data analysis, data interpretation, writing of the report, or the decision to submit for publication. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Results

Enrolment started on May 18 and finished on September 9, 2020, with 45 participants recruited among the 4 participating hospitals. On September 29, the last scheduled visits were completed. As planned, 30 were randomized to the IVM group and 15 to the untreated control group. Two subjects withdrew consent in the IVM group; in 1 case due to a mild rash and nausea after 1 dose of IVM and the other due anxiety; in both cases, the adverse events were judged as possibly related to IVM and resolved spontaneously; the remaining 28 subjects in the IVM group completed treatment. All doses of IVM were completed during hospitalization. One case in the control group was withdrawn from the study due to the initiation of lopinavir on day-5 due to disease progression. All patients assigned to the treatment group started treatment, therefore all subjects were included in the safety analysis (figure 1). One patient in the control group was lost to follow-up after the visit on day-7.

Baseline characteristics showed a mean age of 40·89 (SD 12·48) among study participants, without differences between groups; sex distribution was 20 (44%) females and 25 (56%) males (table 1). Comorbidities and disease stages were similar between groups; with the most frequent comorbidity being obesity in both groups, followed by hypertension, diabetes and chronic obstructive pulmonary disease (table 1). The number of days between symptoms onset and enrolment had a mean (SD) of 3·6 days (1·4) in the control group and 3·5 (1) in the treated group, without significant differences between groups nor cases enrolled beyond the eligibility criteria of up to 5 days since symptoms onset. No major differences in clinical symptoms, signs, or laboratory parameters were observed between groups at baseline (table 1). Disease progression was registered in 3 (7%) of the study population; 2 in the treated group and 1 in among the controls, with 1 case in the IVM group requiring invasive mechanical ventilation and no significant differences in clinical evolution at day-7 between groups.

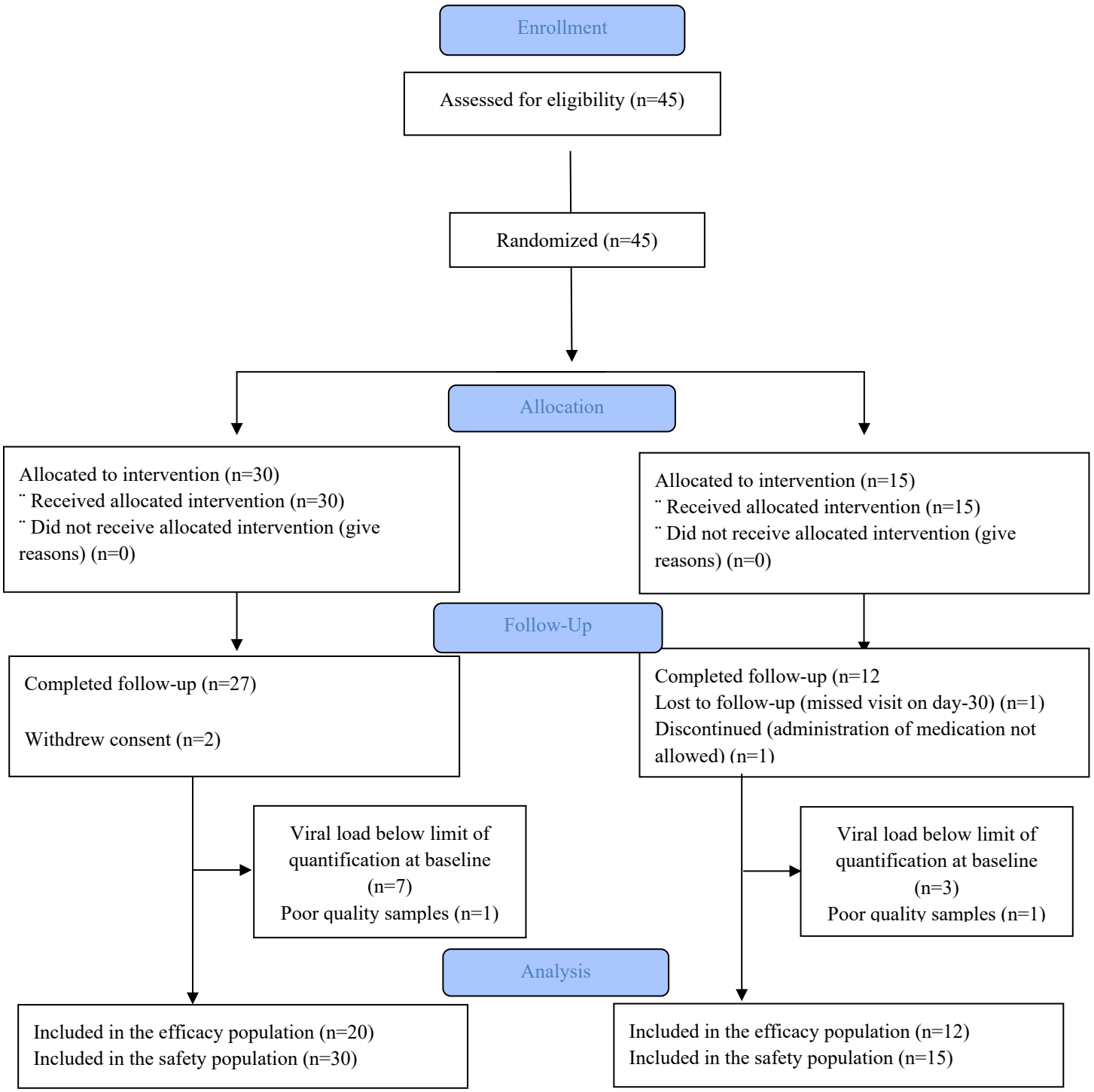
Quantitative viral load at baseline of the whole study population had a median (IQR) of 3·69 log_{10} copies/reaction (1·97-5·74), including 5 (11%) undetectable cases and another 5 (11%) detectable although with <15 copies/reaction (<1·18 log_{10} copies/reaction) at baseline, all these cases remaining undetectable in most samples through the follow-up and excluded from the efficacy analysis. No differences in viral load were detected between males and females. Another 4 cases including 2 with poor quality samples and 2 with early withdrawal were also excluded. The remaining 32 cases (20 treated and 12 control) constitute the efficacy analysis population (fig 1). Baseline viral load in this population had a median (IQR) of 5·59 log_{10} copies/reaction (4·75 - 6·12) in the control group and 3·74 log_{10} copies/reaction (2·8 - 5·79) in the treatment group (p=0·08) (table 1). Viral load dynamics changed similarly in both groups over the study period without significant differences and decreasing over time (figure 2).

The estimation of IVM systemic exposure in treated patients was based on the collection of blood samples at 4-hours after each of the first 3 doses, after the 5th, and at 48 hours after the last

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administered dose. This time-sampling design aimed at measuring the predicted IVM plasma peak plasma concentration (Cmax) and estimating the pattern of drug elimination from the body.

Figure 1: Trial profile



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	Control (n = 15)	Ivermectin (n = 30)
Age (year)	38·1 ± 11·7	42·3 ± 12·8
Gender		
Female	5 (33%)	15 (50%)
Male	10 (67%)	15 (50%)
Weight (kilogram)	79·7 ± 14·4	75·3 ± 15·0
Overweight	8 (53%)	6 (20%)
Obesity I	2 (13%)	11 (47%)
Obesity II	1 (7%)	1 (3%)
Obesity III	1 (7%)	1 (3%)
Oxygen saturation <94%	0	1 (3%)
Log viral load (log10 copies/reaction)	5·39 ± 1·56 (n = 12)	4·18 ± 1·60 (n = 20)
Hematology		
White blood cell count (cell/µL)	4857 ± 1874	6014 ± 2402
Lymphocyte count (cell/µL)	1478 ± 266	1744 ± 747
Biomarkers		
Lactate dehydrogenase (IU/L)	460 ± 117	468 ± 140
Ferritin (mg/dL)	1318 ± 1969	1071 ± 1304
D-dimer (µg/mL)	1·5 (0·1-2·8)	1·5 (0·5-1·8)
Time from symptoms onset (day)	3·6 ± 1·4	3·5 ± 1·0
Body temperature ≥37.5ºC	1 (7%)	4 (13%)
WHO-ordinal scale		
3	13 (87%)	29 (97%)
4	2 (13%)	1 (3%)
Ground glass opacities in thoracic imaging	6 (40%)	14 (47%)
Comorbidities		
Hypertension	3 (20%)	3 (10%)
Diabetes	1 (7%)	6 (20%)
Chronic lung disease/Asthma	1 (7%)	4 (13%)

Table 1. Baseline characteristic of the study population

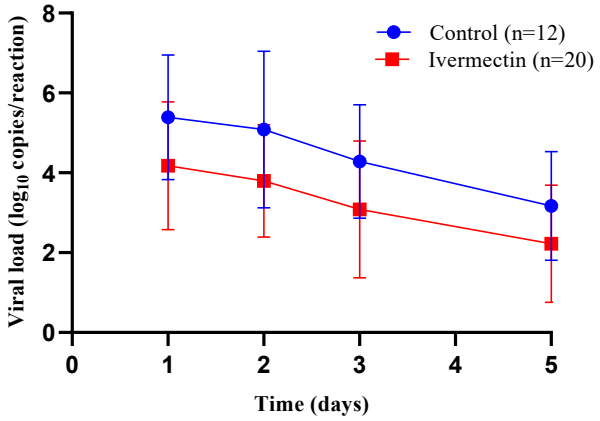
Numeric variables are reported as median (IQR)mean ± standard deviation. Categoric variables are reported as counts (%). Overweight: Body mass index (BMI) 25-29·9 kg/m^2; Obesity I: BMI 30-34·9 kg/m^2; Obesity II: BMI 35-39·9 kg/m^2; Obesity III: BMI >40 kg/m^2. No statistical differences were observed in any of the reported parameters between groups

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Viral load reduction between baseline and day-5 was also similar between groups (figure 2). When mean plasma IVM concentration levels were analyzed in relation to reduction in viral load, a significant positive correlation was identified, with those patients achieving higher mean plasma concentrations of IVM reaching higher reductions in viral load in nasopharyngeal secretions (r: 0·44; p<0·04). This correlation was stronger when the reduction in viral load was related to the IVM exposure corrected by viral load at baseline (r: 0·60; p<0·004). The mean IVM plasma concentration levels also showed a positive correlation with the viral decay rate (r:0·47, p=0·02).

Figure 2. Viral load by quantitative RT-PCR on upper respiratory tract secretions since treatment initiation in patients receiving IVM 0·6 mg/kg/day for 5 days versus untreated controls. Data are mean (SD). Day-1 indicates

.



baseline measurements.

Based on the observed antiviral response, treated patients were divided in two subgroups where 160ng/ml was the threshold plasma concentration. Median Cmax in this subgroup was 202 ng/ml (IQR: 167-268 ng/ml) in the >160ng/ml subgroup and 109 ng/ml (IQR: 91-141 ng/ml) in the <160 ng/ml subgroup (p<0·0001). To further explore this PK/PD relationship, viral load dynamics and reductions between baseline and day-5 were analyzed in the 2 subgroups of IVM treated patients (figure 3a), with median (IQR) reductions in viral load of 42% (31-73) in the control group, 40% (21-46) in treated patients with <160ng/mL median plasma concentrations,

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and 72% (59-77) in the higher concentration group (Kruskal-Wallis p=0·0096), with a statistically significant differences between the latter and the other groups (figure 3b).

Drug-induced effects on viral clearance were also assessed using viral decay rates as an endpoint parameter and its relationship with drug exposure. The viral load decay rate in treated patients with IVM plasma levels >160ng/mL was significantly greater (median 0·64 d^{-1}) compared to that calculated in the untreated control group (median 0·13 d^{-1}) and also to the subgroup with <160ng/mL median plasma concentrations (median 0·14 d^{-1}) (p=0·041) (figure 4a). Similar differences in decay rate were observed comparing controls and subgroups of patients with ratio AUCivm/AUCvl >50, and ratio AUCivm/AUCvl < 50 (p=0·0006) (figure 4b). No significant differences in baseline viral load were observed between IVM concentration subgroups. The relationship between IVM concentration did not correlate with body weight (r^2=0·1) or body mass index (r^2=0·07) among the 28 patients that completed treatment with IVM.

Adverse events were reported in 18 (40%) of the 45 patients included in the safety analysis, which by the design of the study was unblinded; 13 (43%) in the IVM group and 5 (33%) in the control group (table 2). The most frequent adverse event and the only experienced by more than 1 case in the IVM group was rash in 3 (10%) cases (all mild, self-limited and lasting approximately24 h); in the control group, single events of abdominal pain, dizziness, anxiety, anguish, and hyperglycemia (all mild) were reported. A single serious adverse event (SAE) was reported in the trial in a patient in the IVM group with hyponatremia, which has been recently recognized in case series of COVID-19 cases and has not been reported in association to IVM use.[17] A relationship between IVM systemic concentration and adverse events failed to reveal a link between these variables.

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Figure 3. Viral load by quantitative RT-PCR on upper respiratory tract secretions since treatment initiation (mean and SD) (A) and viral load reduction between baseline and day-5 (median and IQR) (B) in untreated controls and IVM treated patients discriminated by their median IVM plasma concentrations. All treated patients receiving IVM 0·6 mg/kg/day for 5 days. Day-1 indicates baseline measurements.

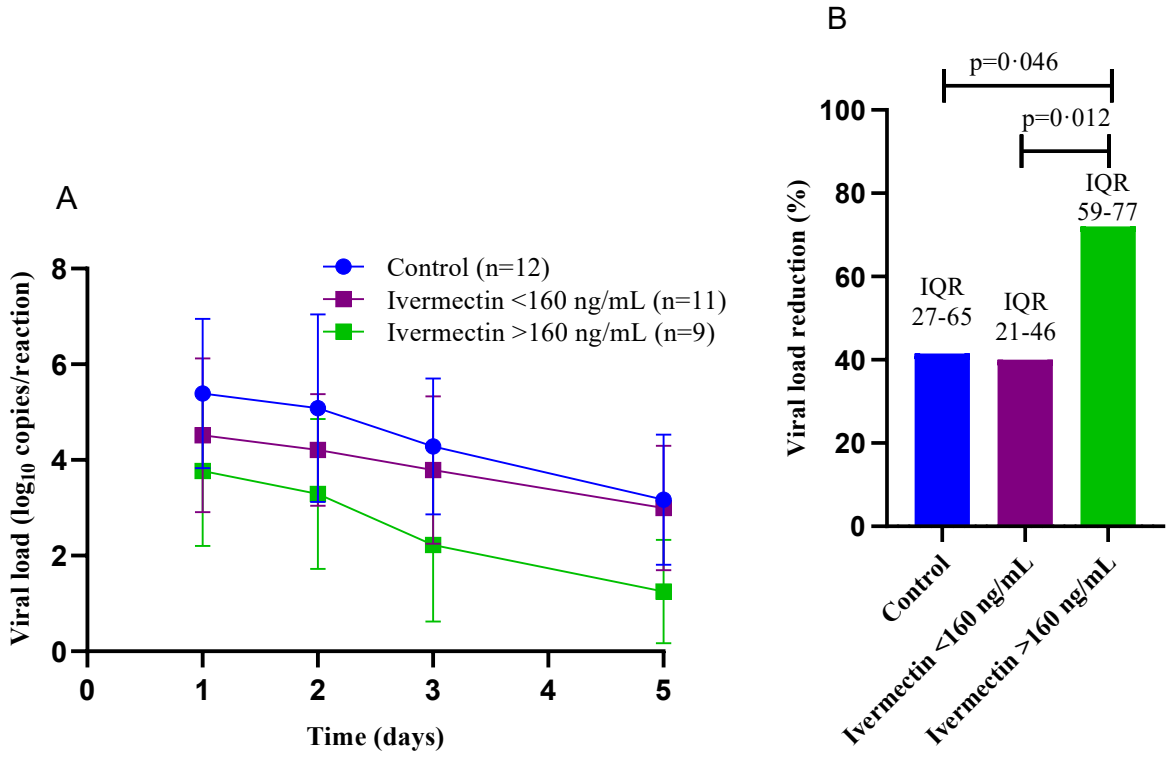
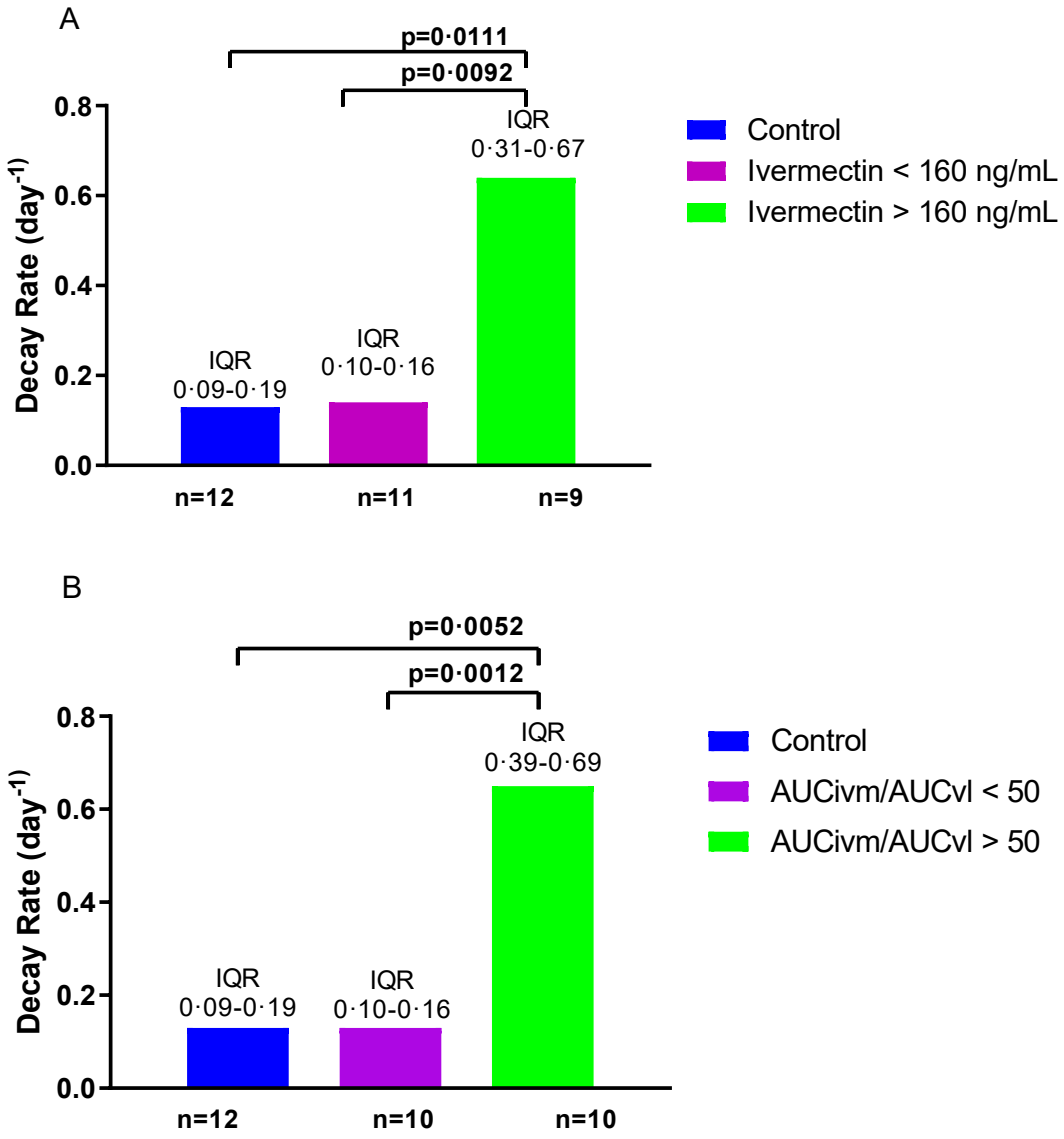


Figure 4. Viral load decay rates by quantitative RT-PCR on upper respiratory tract secretions in untreated controls and IVM treated patients according to median plasma concentrations of IVM (A) and by the ratio between the area under the IVM plasma concentration curve and the area under the viral load curve (AUCivm/AUCvl) (B). Data are expressed as median (IQR).

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	Control (n=15)	Ivermectin (n=30)
Patients with AEs	5 (33%)	13 (43%)
Patients with possible/probable related AEs	NA	9
Patients with SAEs	0	1*

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Patients with possible/probable related SAEs	0	1
Number of AEs	5	17
Number of possible/probable related AEs	NA	11
Number of AEs Grade 3/4	0	3**

Table 2. Summary of events in safety population. AE: adverse event. SAE: serious adverse event. *: hyponatremia; **: include the SAE (hyponatremia) and ALT and AST elevation, both in the same patient.

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Discussion

The results of this pilot clinical trial indicate a concentration-dependent antiviral activity of IVM over SARS-CoV-2 infected patients with mild and moderate disease stages treated within 5 days of symptoms onset. This is the first clinical trial that confirms previous in vitro activity shown in Vero-hSLAM cell cultures.[5] The relevance of IVM plasma concentrations as a surrogate indicator of drug exposure in the sites of viral replication such as nasopharyngeal mucosa and lung tissues, confirms theoretical models stating the need for higher doses than usual to achieve *in vivo* antiviral activity; however, contradict those concerns stating that those drug concentrations would not be achievable at doses with an adequate safety profile.[6,18] A large IVM distribution into different tissues has been demonstrated in different animal species. The extensive pattern of IVM distribution to lung tissue has been well characterized in cattle with a lung tissue to plasma ratio of 2·67.[12] Considering that similar volumes of distribution have been reported for IVM in both cattle and humans and the systemic availability observed in treated patients in this clinical trial, it is reasonable to estimate median IVM levels >395 ng/g in lung tissue. A similar pattern of IVM distribution to lung tissue has been recently simulated using a minimal physiologically based PK model.[19]

The beneficial antiviral effect was seen in the viral load reduction between baseline and day-5 and in viral decay rates only after IVM plasma concentration measurements allowed discrimination between patients achieving higher levels and identifying a strong direct relationship between drug systemic exposure and parameters of viral elimination. Using the threshold of 160 ng/mL for IVM plasma concentrations as an indicator of adequate systemic exposure, statistically significant differences compared to untreated controls were demonstrated even after adjusting for baseline viral load, with those falling below 160 ng/mL reaching viral elimination parameters similar to the control group. Additionally, relevant conclusions on the natural history of the illness can be derived from the behavior of the control group in this trial, which demonstrates the self-limited nature of viral load in SARS-CoV-2 infections, that in 22% of the cases was already extremely low or undetectable at baseline; a finding similar to what has been observed in another trials [20], highlighting the relevance of adequate timing of implementation of antiviral treatment, a critical issue in the design of clinical trials of potential antiviral therapies.

IVM plasma concentrations >160ng/mL were measured in 9 (45%) patients included in the efficacy analysis population. In another trial using a 3-day regimen of 0·6mg/kg for mosquitocidal activity and malaria control among adults, median Cmax (CI95%) was 119ng/mL (45-455).[8] Diet is a key variable affecting the oral bioavailability of IVM, with increased plasma concentrations achieved with fed state, mostly related to the fat content of the meal; still other variables probably play significant roles since IVM is characterized by a high intra and interindividual variability in key PK parameters .[21,22] The interaction of IVM with ABC transporters as P-glycoprotein [23] and the modulation of P-glycoprotein activity after oral

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administration is well known.[24] Thus, variable constitutive and/or induced level of expression and activity of intestinal P-glycoprotein in treated patients, may have contributed to the observed large variability in the pattern of IVM absorption and systemic exposure.

Safety and efficacy of high dose IVM are a subject of research in view of its potential role in increasing clinical usefulness in new indications as is the case of malaria and also aiming for simplified fix-dose rather than weight-based strategies as in Mass Drug Administration campaigns against different Neglected Tropical Diseases. Although further information is needed, this trial adds evidence on the safety of multiple-day high-dose regimens of IVM, without unexpected findings.

Limitations of this trial include a small sample size to detect clinical outcomes beyond antiviral activity, being the latter the critical question this trial was designed to answer. The finding of 2 distinct populations regarding mean IVM systemic concentrations was identified despite the body weight-based dosing and the indication of administering the drug tablets with meals. The lack of a proper registry of the actual content of the meals ingested around the intake of each treatment may add a source of variation to the observed IVM plasma profiles among treated patients.

The assessment of the effect of drug candidates against viruses causing acute respiratory infections is hampered by several aspects of these host-pathogen relationships including short incubation periods, relatively rapid immune control of viral replication, and the high variability in symptom scores among patients.[25] For that reason, key components for adequate endpoints in these trials are sensitive quantifiable measurements of the underlying cause of disease as the quantitative RT-PCR with a wide dynamic range, to control high inter-individual variability in natural infection. [26] Still in this trial, the addition of an integrated assessment taking into consideration drug PK parameters was necessary to identify the potential clinical utility of IVM in SARS-CoV-2 infections. In IVM treated patients, AUCivm/AUCvl ratios >50 were associated with significantly higher viral decay rates. As it has been proposed in an influenza model of antiviral candidate drugs evaluation [25], viral decay rates proved to be a critical parameter of antiviral activity. Additionally, as it has been clearly demonstrated for acute viral infections, early treatment initiation plays a critical role in antiviral activity.[26,27] The potential clinical relevance of these findings remains to be confirmed in trials with clinical endpoints. Beyond clinical aspects of the illness, lowering viral burden might influence infectivity, although there is conflicting data regarding the relationship between burden of viral shedding and infectivity. [28]

Drug repurposing has focused great attention on the search for viable treatments in the context of COVID-19 pandemic, allowing reduced development time and cost. In addition, the use of host-based antiviral compounds has been proposed as an alternative strategy over direct antivirals, that could overcome limitations derived from drug resistance or viral mutations. [29] The proposed antiviral mechanism of IVM is through its ability to inhibit the nuclear import of viral proteins

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mediated by IMPα/β1 heterodimer [5], and it has also been suggested that IVM could promote defense mechanisms such as pyroptosis in infected epithelial cells.[30]

In summary, our findings support the hypothesis that IVM has a concentration dependent antiviral activity against SARS-CoV-2 and provides insights into the type of evaluations to be considered in the assessment of antiviral drugs for the control of COVID-19. Follow-up trials to confirm our findings and to identify the clinical utility of IVM as either sole intervention or in combination with other tools for the control of the ongoing pandemic outbreak are warranted.

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Coordination: ROC, ES, AM, CL, DA, AK
Supervision of the project: AK, CL, SG

Declaration of interests

AK reports grants from Laboratorio Elea/Phoenix. MAT, MDG and ES are employees of Laboratorios Elea/Phoenix. SG is a moember of the Board of Directors of Laboratorio Elea/Phoenix. All other authors declare no competing interests.

Data sharing

De-identified individual clinical and laboratory data and a data dictionary; will be made available to others after 3 months of trial publication upon request to the corresponding authors, only for research, non-commercial purposes to individuals affiliated with academic or public health institutions.

Acknowledgments: We thank Romina Guelho for technical assistance. Natalia Pacífico, Belén Bargallo, Julián García, Andrés Benchetrit, Guillermo Onis, Luis Vanzetti, Liucó Zubeldía Brenner, Natalia Díaz for patient care. Javier Toibaro for assistance in statistical análisis. Graciela Ciccia, Ignacio Demarco and Alicia Bagnoli for assistance in Project support.This work was supported by grant IP-COVID-19-625 from Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación, Argentina and Laboratorio ELEA/Phoenix, Argentina.

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This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3714649

frontiers in Pharmacology

FOI 26-3132 - Document 5
REVIEW
published: 06 October 2020
doi: 10.3389/fphar.2020.582025

Available Compounds With Therapeutic Potential Against COVID-19: Antimicrobial Therapies, Supportive Care, and Probable Vaccines

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The recent outbreak of the COVID-2019 (coronavirus disease 2019) due to the infectious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has realized the requirement of alternative therapeutics to mitigate and alleviate this lethal infection. These alternative therapies are effective when they are started at the initial stage of the infection. Some drugs that were used in previous other related infections SARS-CoV-2003 and Middle East respiratory syndrome coronavirus (MERS-CoV)-2012 could be potentially active against currently emerging SARS-CoV-2. This fact imparts some rationale of current interventions, in the absence of any specific therapeutics for SARS-CoV-2. It is imperative to focus on the available antimicrobial and adjunct therapies during the current emergency state and overcome the challenges associated with the absence of robust controlled studies. There is no established set of drugs to manage SARS-CoV-2 infected patients. However, closely following patients' conditions and responding with the dosage guidelines of available drugs may significantly impact our ability to slow down the infection. Of note, it depends upon the condition of the patients and associated comorbid; therefore, the health workers need to choose the drug combinations judiciously until COVID-19 specific drug or vaccine is developed with the collective scientific rigor. In this article, we reviewed the available antimicrobial drug, supportive therapies, and probable high importance vaccines for the COVID-19 treatment.

Keywords: antimicrobial therapy, coronavirus disease 2019, drug, severe acute respiratory syndrome coronavirus 2, supportive therapy, vaccine

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Edited by:
Mariusz Skwarczynski, The University of Queensland, Australia

Reviewed by:
Zahid Hussain, University of Sharjah, United Arab Emirates
Pio Maria Furneri, University of Catania, Italy

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Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *10 July 2020*
Accepted: *15 September 2020*
Published: *06 October 2020*

Citation:
Kumar R, Srivastava JK, Singh R, Siddiqui MH, Mansouri RA, Abdulhakim JA, Bin-Jumah MN, Alkahtani S, Abdel-Daim MM and Uddin MS (2020) Available Compounds With Therapeutic Potential Against COVID-19: Antimicrobial Therapies, Supportive Care, and Probable Vaccines. Front. Pharmacol. 11:582025. doi: 10.3389/fphar.2020.582025

INTRODUCTION

Coronavirus is named after the crown-like structures projected from the envelope of its surface. Coronavirus belongs to the family Coronaviridae, which comprises of four genera: α-coronavirus, β-coronavirus, δ-coronavirus, and γ-coronavirus. α and β-coronaviruses infect mammals; however, γ and δ-coronaviruses mostly infect birds. The highly contagious coronavirus disease 2019 (COVID-19) belongs to the genus β-coronavirus (Loeffelholz and Tang, 2020; Zhu et al., 2020). It is caused by a novel β-coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak that has by far affected almost all the countries amounting to the death of about 850,000 individuals till date internationally.

The SARS-CoV-2 contains four structural proteins, namely, spike, membrane, envelope, and nucleocapsid (Bosch et al., 2003). The transmembrane spike proteins are trimeric glycoproteins present on the surface of the virus. These proteins are responsible for diversity and host-tropism of the virus. The virus attaches to the angiotensin-converting enzyme 2 (ACE2) (functional host-receptors for SARS-CoV-2), and through membrane fusion or endocytosis enters the host cell (Li et al., 2003; Chen Z. et al., 2020). In the alveolar space, ACE2 has highly expressed an upper side of lung epithelium cells, and therefore, SARS-CoV-2 can easily enter and abolishes these cells (Hamming et al., 2004; Jia et al., 2005; Zou et al., 2020). The released viral genetic material (single-stranded positive-sense ribonucleic acid) reaches the nucleus and starts the self-replication, transcription, and translation. The biosynthesis of viral proteins in the host-cells enables it to assemble, mature, and get released as new copies of viral particles.

The SARS-CoV-2 primarily infects respiratory system but other organs like kidney, heart, ileum, and spleen can also be infected (Kabir et al., 2020). The common symptoms of this virus infection include dyspnoea, dry cough and fever (Huang et al., 2020). However, generalized weakness, diarrhoea, vomiting, headache, and dizziness may also be observed (Shi et al., 2020). There is currently no specific therapeutics available for the prevention or cure for SARS-CoV-2 as per the Food and Drug Administration (FDA), the World Health Organization (WHO), and the Centers for Disease Control and Prevention (CDC). The WHO guidelines emphasized the role of supportive care on the basis of illness severity, i.e., symptomatic treatment for mild infections and evidence-based ventilatory management for acute respiratory distress syndrome and early recognition and treatment of bacterial infections and sepsis in severe COVID-19 cases (World Health Organization, 2020). So far, agents under clinical trials are also being used based on their *in vitro* activity against SARS-CoV-2. However, there are minimal clinical evidence could be collected so far, and the efficacy of such drug therapies in the treatment of SARS-CoV-2 has not been established. The vaccines against COVID-19 will probably take at least a year to become available, and the use of available drugs to prevent disease (chemoprophylaxis) is the main option in hand for the management of infected patients.

Therefore, for the management of the SARS-CoV-2, the current treatments are entirely supportive by nature. In general, pharmacological treatment is not suggested for young patients with mild indications and without other underlying chronic conditions (Gao et al., 2020). **Figure 1** shows the life cycle of SARS-CoV-2 along with its possible inhibitors at various stages of its attachment, multiplication, and growth in the host cell. Available antimicrobials, adjunctive, supportive therapies, and probable vaccines are discussed in the following sections. Dugs and support therapies that are currently under clinical are listed in the **Supplementary Material**.

ANTIMICROBIAL THERAPIES AGAINST COVID-19

Chloroquine

Chloroquine (**Figure 2**) has been used for years against malaria. This cost-effective and widely available therapeutic agent is also a robust antiviral, as it blocks a virus from invading the human cells. A large number of research groups are currently reviewing whether it effectively decreases the viral load in patients with SARS-CoV-2 (Savarino et al., 2006; Marmor et al., 2011; Cortegiani et al., 2020). However, *in vitro* and limited clinical data indicate therapeutic advantages against SARS-CoV-2 infection. SARS-CoV-2 is an enveloped virus, and it enters the cell by endocytosis. Endocytosis is a cellular process in which elements are taken into the cell by making a tiny vesicle. Once inside, a dip in pH promotes the fusion of the virus envelope with the membrane of the vesicle that encompasses it, to be released into the cytoplasm (McChesney, 1983). Till date, chloroquine is verified effective against COVID-19 *in vitro* through influencing bis (monoacylglycero) phosphate entry by controlling the endocytic pathway (Carrière et al., 2020). The use of chloroquine against COVID-19 might be a risk and require continuous monitoring until confirmed clinical evidence are available.

Hydroxychloroquine

Hydroxychloroquine is also classified under the antimalarial agent. It is a comparatively less toxic analog of chloroquine, which has already been found to stop the replication of SARS-CoV-2 *in vitro* in cell culture studies (Colson et al., 2020; Yao X. et al., 2020). It exhibits the mechanism of action, similar to the chloroquine for COVID-19 patients. It primarily increases the pH in the endosomes, thereby, stops their acidification and maturation and interferes with the entry of the virus in the cell (Hacker et al., 1998; Macfarlane et al., 1998; Vincent et al., 2005; Christophe et al., 2006). Also, it inhibits the toll like receptors (TLRs) and thereby reduces the pro-inflammatory cytokines Interleukin-6 production (Fox, 1993; Fox, 1996; Ben-Zvi et al., 2012; Jorge et al., 2018; Sahraei et al., 2020; Wu et al., 2020a).

Hydroxychloroquine is being used as a potential therapeutic agent for the treatment of SARS-CoV-2 infected patients under the FDA-Emergency Use Authorization (EUA) by following the same guidelines as recommended for chloroquine. However, it is not an FDA-approved drug for the treatment of SARS-CoV-2 infection. Hydroxychloroquine shows better *in vitro* efficacy

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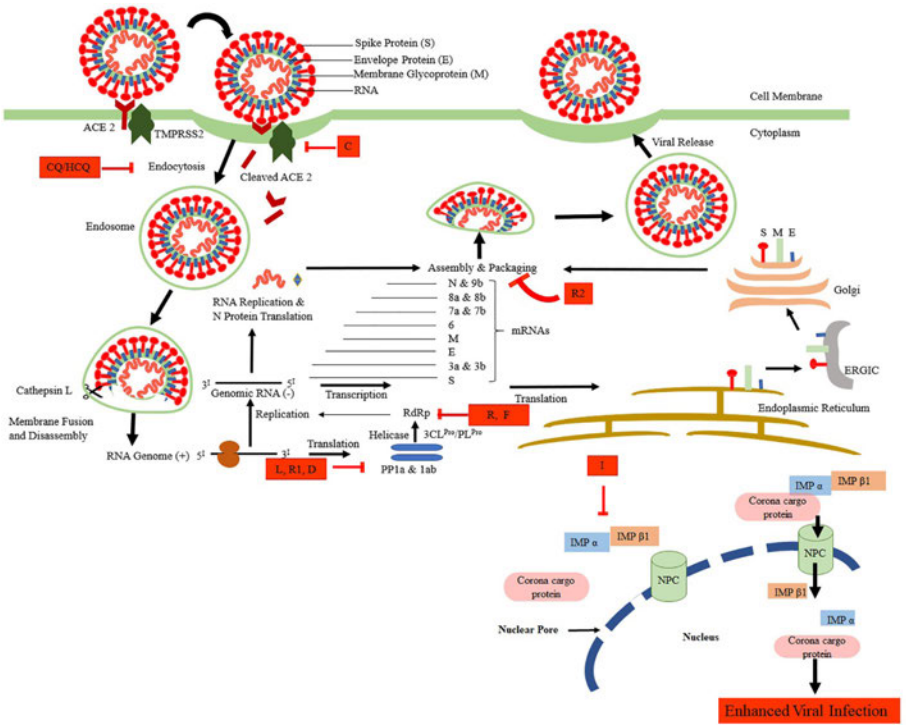


FIGURE 1 | The life cycle of SARS-CoV-2 and its possible inhibitors. SARS-CoV-2 enters the target cells *via* an endosomal pathway. The S protein binds with the angiotensin-converting enzyme 2 (ACE2) of the cell primed by serine protease TMPRSS2. Viral RNA is unveiled in the cytoplasm to produce PP1a and PP1ab polyproteins, which are cleaved to form non-structural proteins. These non-structural proteins facilitate the formation of negative RNA by the process of replication and transcription. This, in turn, translates to N protein, another set of translation takes in the endoplasmic reticulum-ERGIC-Golgi to produce structural proteins (S, M, and E). Finally, the packing of viral RNA with N proteins and further assembly of S, M, and E proteins take place to form SARS-CoV-2 buds, which are released from the infected cell by exocytosis. Various drugs that are shown to inhibit the SARS-CoV-2 at various stages are CQ/HCQ, Chloroquine/Hydroxychloroquine; L, Lopinavir; R1, Ritonavir; I, Ivermectin; R, Remdesivir, R2, Resveratrol; D, Darunavir; C, Camostat.

against SARS-CoV-2 as compared to the chloroquine. Effective concentration-50 (EC50) values of the hydroxychloroquine were reported to be 6.25 μM at 24 h and 5.85 μM at 48 h as compared to chloroquine, which is about 100 μM at 24 h and 18.01 μM at 48 h. Moreover, hydroxychloroquine has comparatively better compatibility with other antiviral agents e.g., lopinavir, ritonavir, ribavirin, oseltamivir, remdesivir, etc., which could be used concomitantly during COVID-19 treatment (Wang et al., 2020). In addition, it does not interfere with intravenous immunoglobulins and interferons. Although there are some common adverse drug reactions of hydroxychloroquine such as nausea, stomach pain, vomiting, headache, and in some cases, itching, they do not require discontinuation of the drug.

Prolonged use of the drug may lead to some significant side effects irregular heartbeats, yellowing of the eyes, convulsions or seizures, retinopathy, blurred vision, muscle weakness, difficulty hearing, bruising, or bleeding of the skin, etc. (Ling Ngan Wong et al., 2008; Marmor et al., 2011). Therefore, risk and benefit balance assessment must be done while recommending the hydroxychloroquine in such cases. There are *in vitro* and clinical results in support (Gautret et al., 2020; Yu et al., 2020) and against (Cavalcanti et al., 2020; Chen J. et al., 2020; Geleris et al., 2020; Molina et al., 2020; Rosenberg et al., 2020) the use of hydroxychloroquine for COVID-19 patients. Of note, the data available so far suggest that therapeutic efficacy of HCQ in COVID-19 is well overshadowed by its ineffectiveness and detrimental effects as shown by some of the above-referred studies. Therefore, it is suggested to avoid use of HCQ for COVID-19 patients (unless necessary) until clear, sufficient, well-conducted, randomized, and controlled clinical data is available to reach the right conclusions.

Lopinavir and Ritonavir

Lopinavir and Ritonavir (**Figure 3**) are HIV Protease inhibitors. Animal model and in- vitro studies suggest that lopinavir and ritonavir have therapeutic activity for SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), but their role in the treatment of SARS-CoV-2 is not clear (Chen et al., 2004; Yao T. T. et al., 2020). These drugs may bind to the Mpro, which is a vital enzyme for the SARS-CoV-2 replication. Mpro facilitates proteolytic processing, which is released by auto-cleavage of pp1a and pp1ab. Then Mpro, in turn, cleaves pp1a and pp1ab to release functional proteins necessary for viral replication (Adedeji et al., 2014; Liu et al., 2020). A retrospective cohort study was done to review the clinical course, and risk factors for mortality during lopinavir-ritonavir

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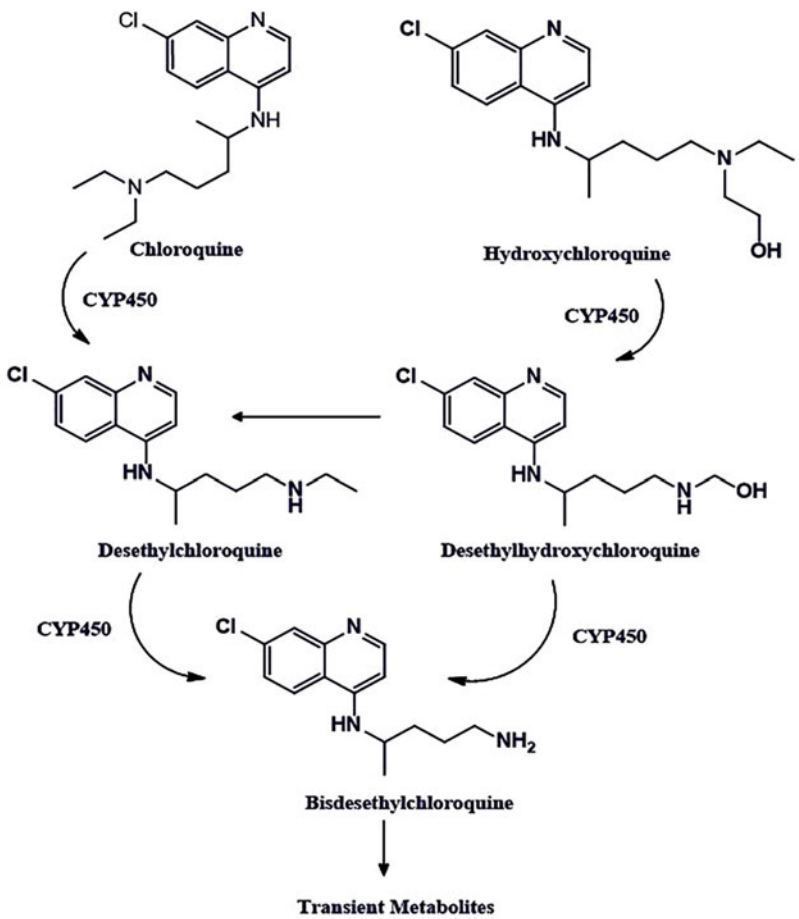


FIGURE 2 | The chemical structures of chloroquine and hydroxychloroquine. The chloroquine contains the N-diethyl group, whereas hydroxychloroquine has an N-hydroxyethyl group at the same position, which is the only structural difference these two drugs have. Both drugs share bis-desethylchloroquine as a common metabolite.

therapy of the SARS-CoV-2 infected hospitalized patients (n=29). There was no difference found in the virus shedding duration with lopinavir-ritonavir (Zhou F. et al., 2020).

As per another recent randomized, open-label, controlled trial (n=199) studies, lopinavir-ritonavir treatment applied to standard supportive care did not result in clinical improvement or mortality in severely infected patients with SARS-CoV-2 as compared to the patients on standard supportive care only. However, the median time to the clinical improvement was found to be reduced by one day with lopinavir-ritonavir treatment (Cao et al., 2020). This modest improvement still seems to be significant because the study was carried out on the severely infected patients with a mortality rate of 22.1% as compared to the 11%–14.5% as reported in preliminary descriptive studies of hospitalized SARS-CoV-2 infected patients (Chaolin et al., 2020; Chen N. et al., 2020; Osborne et al., 2020). After analyzing the recently published clinical outcomes, it was found that the clinical improvement time was not significantly reduced for SARS-CoV-2 infected patients upon the use of lopinavir-ritonavir as compared to standard care. The study by Cao et al. (2020) demonstrating lower acute respiratory distress syndrome (ARDS) with lopinavir-ritonavir and less adverse drug reactions as compared to the standard care. Overall, the effectiveness of lopinavir-ritonavir in severe SARS-CoV-2 could not be considered very encouraging until more conclusive clinical data is available.

Further, trials with the optimal sampling methods and specimen from the lower respiratory tract from the mild to severe SARS-CoV-2 infected patients may lead to better conclusions for lopinavir-ritonavir treatment. More clinical trials (NCT04261907, NCT04321174, NCT04330690, ChiCTR2000030187) data for evaluating the therapeutic efficacy of lopinavir-ritonavir in COVID-19 are awaited.

Ivermectin

Ivermectin (**Figure 4**) is a broad-spectrum antiparasitic drug but shown promises of antiviral activity in recent *in vitro* studies (Canga et al., 2008; Wagstaff et al., 2012; Lundberg et al., 2013; Tay et al., 2013; Götz et al., 2016). Importin (IMP) α/β1 is a heterodimer which facilitates the nuclear import of coronavirus

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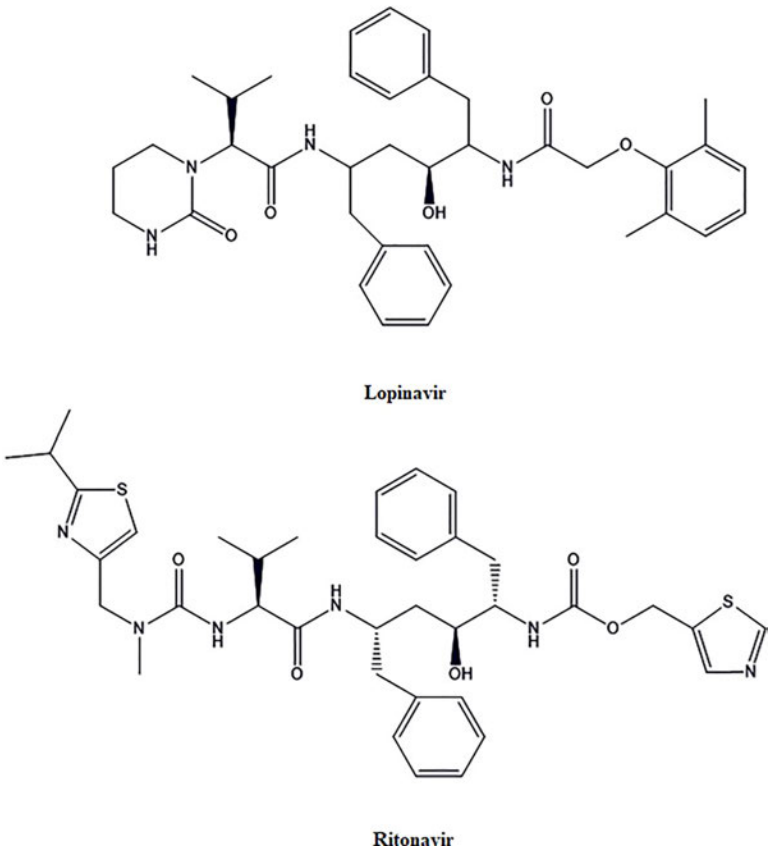


FIGURE 3 | The chemical structures of lopinavir structure of ritonavir.

cargo protein, present in the cytoplasm of the infected cell. It translocates coronavirus protein through the nuclear pore complex (NPC) inside the nucleus where the complex gets separated. The cargo could diminish the antiviral response of the infected cell, leading to increased infection. Ivermectin binds to Impα/β1 heterodimer, thereby destabilize and prevents Impα/β1 from interacting with viral protein. It leads to the stoppage of the translocation of the viral protein into the nucleus. This mechanism is further substantiated by various studies to demonstrate the effect of ivermectin on RNA-virus infections, e.g., human immunodeficiency virus-1 (HIV-1) (Wagstaff et al., 2011), Venezuelan equine encephalitis virus (VEEV), influenza, West Nile Virus (Yang et al., 2020), and Dengue virus (DENV) 1-4. This is due to the dependence of these RNA viruses on IMPα/β1 during infection (Caly et al., 2012; Jans et al., 2019).

Of note, the FDA has only approved this drug for the treatment of parasitic worms such as onchocerciasis and intestinal strongyloidiasis along with some topical formulations for the human to be used upon prescription only for external parasite treatment such as rosacea and head lice. One of the recent *in vitro* studies conducted by Clay et al. (2020) confirmed the antiviral activity of ivermectin against SARS-CoV-2. The SARS-CoV-2 infected Vero/hSLAM cells were subjected to 5 μM ivermectin for 48 h. The study indicates about 5,000-fold decrease in viral RNA as compared to the control. This was the first study to assess and demonstrate the effectiveness of ivermectin in COVID-19. The authors reported that the ivermectin may have antiviral effects by inhibition of IMP α/β receptor, which is responsible for transmission of the viral proteins into the nucleus of host cell. Based on the findings in the study authors proposed clinical studies to further confirm the potential benefits of this drug in the treatment of COVID-19 (Heidary and Gharebaghi, 2020). It is worth mentioning here that ivermectin activity in cell has not been reproduced in the mouse infection models for many viruses. Also, its activity has been not yet proven clinically. The concentration of drug required to be effective in SARS-CoV-2 infected cell culture is in the microgram range but its safe blood levels for imparting therapeutic activity is 20–80 ng/ml. Still, the ivermectin is being available worldwide which may be due to its favorable pharmacokinetics and therapeutic window. Therefore, treatment with ivermectin for COVID-19 patients should be strictly by licensed health workers after a careful review of the patient's health condition until robust and conclusive clinical data (NCT04360356, NCT04343092, NCT04351347) are available.

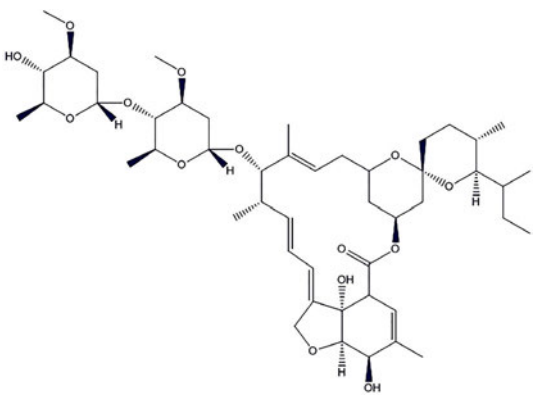


FIGURE 4 | The chemical structure of ivermectin.

Remdesivir

Remdesivir is an investigational nucleoside analog and available only through compassionate use and study protocols. The *in vitro* studies suggest that it exhibits antiviral activity including SARS-CoV-2 (Agostini et al., 2018; Brown et al., 2019; de Wit et al., 2020; Ko et al., 2020; Gordon et al., 2020). Mono-phosphoramidate remdesivir is a prodrug of remdesivir-triphosphate (Warren et al., 2016). Remdesivir (**Figure 5**) is a 1′-cyano-substituted adenosine nucleotide analog that undergoes a metabolic mechanism, activating nucleoside triphosphate metabolite, which inhibits the RNA dependent RNA polymerase (RdRp).

Remdesivir-triphosphate competes with adenosine-triphosphate (ATP) and gets incorporated in the viral RNA. The RNA synthesis terminates after three nucleotides are further added from the position of remdesivir-triphosphate incorporation (Sheahan et al., 2017). The *in vitro* studies suggest that remdesivir exhibits potential therapeutic activity against clinical isolate [Half-cytotoxic concentration (CC50) > 100 mcgM; EC50 = 0.77 mcgM; Selective index (SI) > 129.87] of SARS-CoV-2. One of the recent studies reported that the clinical improvements were observed in 68% (36 out of 53 patients) of the hospitalized COVID-19 patients when treated with remdesivir on a companionate use basis (Grein et al., 2020). However, currently reported studies have some vital limitations such as the small sample size, the relatively less duration of follow-up, potential missing data, the insufficient of information on some patients that were initially involved in the study, and the absence of a randomized control group. Currently, several clinical trials for the evaluation of the efficacy of remdesivir in COVID-19 patients is being conducted (NCT04302766, NCT04323761). More significant and conclusive clinical evidence is expected in the coming months.

Favipiravir

Favipiravir (**Figure 6**) inhibits RNA-dependent RNA polymerase (RdRp). It structurally resembles the endogenous guanine and shows broad-spectrum antiviral activity through competitive inhibition (Furuta et al., 2013). The *in vitro* studies suggest its therapeutic activity against RNA viruses (respiratory syncytial virus, rhinovirus, and, poliovirus) (Furuta et al., 2002; Dong et al., 2020; Shiraki and Daikouku, 2020; Yanai, 2020). Recently, Wang et al. (2020) conducted *in vitro* and demonstrated the effectiveness of favipiravir against SARS-CoV-2. In another open-label control study was done by Cai et al. (2020) to assess therapeutic potentials of favipiravir against SARS-CoV-2. Total of 35 patients were given oral favipiravir with interferon-α aerosol inhalation and a significant reduction in viral clearance time was observed. The multivariable Cox regression analysis further confirmed that the favipiravir was individually associated with faster clearance of the virus. The favipiravir also demonstrated noteworthy improvement in chest imaging and significantly smaller adverse reactions (e.g., nausea, vomiting, etc.) as compared to the lopinavir/ritonavir treated patients. Further, case reports for effectiveness of favipiravir are also reported by the Japanese association for infectious diseases on their web symposium as it was able to improve the clinical symptoms by 90%, 85%, and 61%, in mild, moderate, and severe COVID-19 cases, respectively after 14 days from the start of the treatment (Japanese Association for Infectious Diseases, 2020) Based on the evidence, favipiravir may be a relatively safe and effective drug for COVID-19 at present and expected to emerge as a broad-spectrum antiviral drug against SARS-CoV-2. Its efficacy (NCT04349241, NCT04358549, NCT04346628, NCT04351295, ChiCTR 2000030113) and different combinations with other drugs (favipiravir + hydroxychloroquine: NCT04359615; favipiravir + tocilizumab: NCT04310228; favipiravir + imatinib + telmisartan + hydroxychloroquine: NCT04356495) are being clinically evaluated for effectiveness against COVID-19.

Apart from the above-mentioned antimicrobial agents, there are some drugs such as darunavir, oseltamivir, nitazoxanide, and ribavirin, that are being discussed in the scientific community to have efficacy against SARS-CoV-2. However, due to the absence of clinical evidence or higher risk factors in comparison to the benefits, such drugs are not recommended to be used in COVID-19. Darunavir is an HIV protease inhibitor that is being considered for its therapeutic activity against COVID-19;

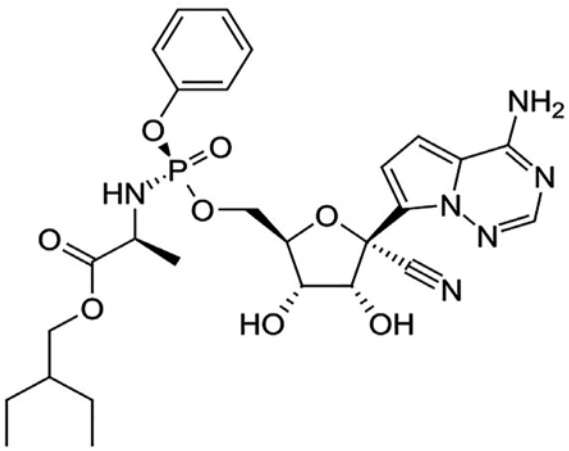


FIGURE 5 | The chemical structure of remdesivir.

FOI 26-3132 Document 5

however, there are no *in vivo* or clinical data available so far, to support the pharmacological efficacy and safety of the drug for COVID-19 patients. The oseltamivir is a neuraminidase inhibitor SARS-CoV-2 does not utilize the neuraminidase enzyme for their budding and multiplication (McKimm-Breschkin, 2012). Therefore, no therapeutic activity is expected by oseltamivir for COVID-19. Nitazoxanide shows some activity against SARS-CoV-2. However, it does not demonstrate any benefit when tested clinically (Gamino-Arroyo et al., 2019).

Moreover, its safety profile and tolerance are also some of the major concerns. Another antiviral agent ribavirin is a guanosine analog that inhibits the replication of DNA and RNA virus. It has shown mixed pharmacological efficacy toward other novel viruses, e.g., SARS-CoV and MERS-CoV (Graci et al., 2006). However, there is no conclusive clinical demonstration of its efficiency toward SARS-CoV-2. Also, the dosage used for its testing for MERS-CoV (800-3600mg/Day) poses a significant adverse reaction profile (Gross and Bryson, 2015; Mo et al., 2016; Arabi et al., 2019). Though, outcomes of some clinical trials for ribavirin in combination with other drugs (ribavirin + lopinavir/ritonavir + interferon-β1b are: NCT04276688, ribavirin+ interferon (IFN)-α/lopinavir-ritonavir/chloroquine phosphate/ arbido: NCT04306497) are still awaited.

ADJUNCTIVE/SUPPORTIVE CARE AGAINST COVID-19

Monoclonal Antibodies

Tocilizumab

It is widely reported that the SARS-CoV-2 activates the innate immunity and the levels of numerous cytokines, such as interleukin (IL)-1β, IL-6, IL-8, TNF, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF), along with chemokines, such as monocyte chemoattractant protein (MCP)1, macrophage inflammatory protein (MIP)1α, and interferon gamma-induced protein (IP10) get increased with higher levels in those who are severely infected (Schett et al., 2020; Zhang C. et al., 2020; Zhang W. et al., 2020). Tocilizumab competitively inhibits the soluble as well as membrane-bound IL-6 receptor and thereby interferes with its diverse mediated signaling process. IL-6 is formed by T-cells, B-cells, monocytes, lymphocytes, and fibroblasts. This pro-inflammatory cytokine, which is associated with various physiological processes, e.g., activation of T-cell and immunoglobulin secretion, acute-phase protein synthesis initiation in the liver, and activation of hematopoietic precursor cell differentiation and proliferation. Tocilizumab is given intravenously in the treatment of COVID-19 in some countries such as China, and Italy resulted in encouraging outcomes (Harrison, 2020). In one of the retrospective reviews, 21 SARS-CoV-2 infected patients were analyzed, when tocilizumab was added to the standard therapy. Clinical symptoms, lymphocyte percentage, computed tomography (CT) opacity changes, and C-reactive protein (CRP) levels were reported to be improved in these patients, which suggests that tocilizumab has clinical benefits (Xu et al., 2020). However, there were no comparators reported in this study.

In another retrospective study, laboratory descriptors of IL-6 and CRP were assessed for prior and after tocilizumab treatment of 15 COVID-19 patients (7 critically ill; 6 seriously ill; 2 moderately ill). The CRP levels rapidly ameliorated in all the patients, and the IL-6 level in serum was found to further increased initially and then reduced [decreased from 126.9 (10.7–257.9) to 11.2 (0.02–113.7) mg/L ($P < .01$)] after tocilizumab therapy in 10 patients. However, 3 patients who received only one dose of the tocilizumab failed to recover (died), and 2 showed disease aggravation (Luo et al., 2020). Although the above-mentioned studies reported a better response of tocilizumab in COVID-19 patients, one should be cautious while evaluating the outcomes as the reported cases and the duration of treatment (ranging from 2–7 days) still seems to be insufficient. Furthermore, the use of laboratory parameters to express disease activity is a difficult task and may influence the outcome of the study. More significant clinical data for the efficacy of tocilizumab in the management of COVID-19 is awaited from ongoing trials (NCT04335071, NCT04356937, NCT04363853, NCT0 4317092, etc.).

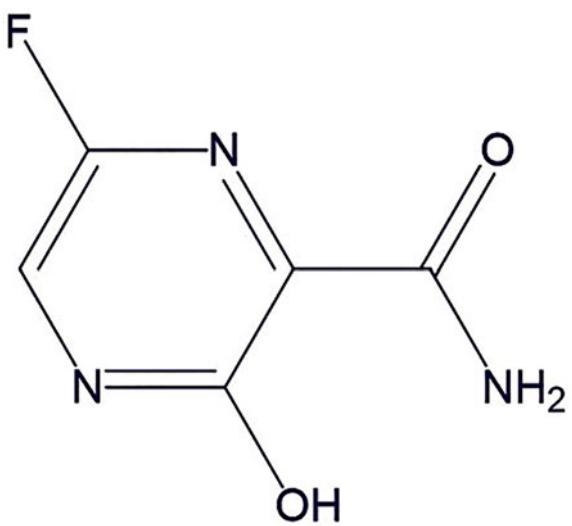


FIGURE 6 | The chemical structure of favipiravir.

Sarilumab

Sarilumab is also an IL-6 receptor inhibitor that interferes with the IL-6 driven through binding with both membrane-bound and soluble IL-6 (sIL-6R and mIL-6R) receptors. The clinical efficacy of sarilumab for COVID-19 patients is under the process of evaluation (NCT04324073; NCT04315298; NCT04327388; NCT04322773; NCT04321993), and the pharmacological potential of it could only be concluded once the robust data evidence are available (Menzella et al., 2020).

Leronlimab

Leronlimab is an investigational humanoid monoclonal antibody and may be used for mitigation of the cytokine storm, which

FOL-26-3132 Document 5

could be a major component in severely diseased COVID-19 patients. It binds with chemokine receptor-5 (CCR5) to alleviate the cytokine release syndrome. The evaluation of leronlimab is now being done in a limited number of severely SARS-CoV-2 infected patients with the emergency investigational new drug application, FDA (CytoDyn, 2020). Data from ongoing clinical trials (NCT04343651, NCT04347239) for the evaluation of therapeutic efficacy and safety of leronlimab will yield a better understanding of its role in the management of COVID-19.

Baricitinib

Baricitinib is a Janus kinase (JAK) inhibitor. Cytokine receptor interactions with cell-membrane produce signals which are further transmitted by the intracellular JAK enzymes to impact the process of immune cell function and hematopoiesis. JAKs phosphorylate and trigger signal transducers and activators of transcription proteins (STATs) during this signal transmission pathway, which affects the intracellular activity. Baricitinib modulates the signaling pathway by preventing phosphorylation of JAKs and thereby inhibiting the STATs activation. The cytokine signal transduction takes place by the pairing of JAKs. Baricitinib has a higher binding affinity for JAK1, JAK2, and tyrosine-protein kinase-2 (TYK2), as compared to JAK3. It inhibits the cytokine activated STAT phosphorylation facilitated by JAKs (JAK1-JAK2, JAK1-JAK3, JAK1-TYK2, or JAK2-TYK2) in the human leukocytes. Baricitinib is an approved drug for rheumatoid arthritis therapy, and if it could pose an antiviral activity by inhibiting JAK-STAT signaling with the same approved dose, that would be a great advantage. However, at the time of early viral infection JAK-STAT signaling pathway (primarily JAK1 and JAK2) activated by interferon gives rise to the increase in the regulation of several interferon-controlled genes that rapidly kill viruses in infected cells (Favalli et al., 2020). To crack this mechanism, many viruses, have developed viral encoded factors to counteract and antagonize the JAK-STAT pathway, which is a crucial descriptor of their viral effect (Fleming, 2016). Therefore, inhibiting the JAK-STAT signaling by baricitinib seems to be substantial facilitation for viral evolution in the host cell.

This argument poses an interesting question upon the general use of baricitinib as an adjunct therapy for COVID-19 patients. However, it is also speculated that patients with the early asymptomatic infection do not need hospitalization, and about 80% of patients can get cured, mostly by endogenous antiviral mechanisms, which certainly includes the interferons (Richardson et al., 2020; Stebbing et al., 2020). Such patients do not require JAK-STAT signaling inhibition. Only the hospitalized patients with after the peak SARS-CoV-2 load (after about 7–10 days of indication onset) show the hyper inflammation due to the so-called cytokine storm resulted from a severe infection, may require the baricitinib therapy. Of note, this clinically critical stage is supported by high level signaling through JAK-STAT pathway, and thereby, enhanced interferons $\alpha+\beta$ and IL-6 levels are reported in the patients. In one of the recent pilot study, 12 moderately SARS-CoV-2 infected patients were found to show improvements in clinical and laboratory parameters with baritinicib (Cantini et al., 2020). In another non-controlled, retrospective cohort study with 15 moderate to severe COVID-19 patients, it was reported that baritinicib with HCQ leads to recovery and clinical improvements (Titanji et al., 2020). However, the efficacy of baritinicib could not be confirmed with scant and insufficient clinical results until enough data about the clinical efficiency and safety profile of baricitinib (NCT04321993, NCT04320277) is available.

COVID-19 Convalescent Plasma

The rationale behind this therapy is that the patient recovered from the COVID-19 may contain antibodies against SARS-CoV-2 in their plasma. Though convalescent plasma (CP) is not intended to prevent COVID-19, it may be used for the severely ill patients at the life-threatening stages as the last resort to improve the survival rate. Of course, the clinical evaluation data for its efficacy must be substantial to prove its effectiveness against the virus. There is evidence that CP works well in reducing the mortality rate of severely infected patients without the occurrence of severe adverse events (Soo et al., 2004; Cheng et al., 2005; Lai, 2005; Chen L. et al., 2020). Moreover, such treatment has worked well with MERS (Arabi et al., 2015) influenza A H1N1 pandemic 2009 virus (Hung et al., 2013), and HIV 1 (Lu et al., 2016). In a timely descriptive study, the effects of ABO-compatible CP therapy were evaluated on 6 COVID-19 positive patients. The evaluation was done based on symptoms improvement, radiologic changes, and laboratory testing. The therapy resulted in the resolution of GGOs and the consolidation of 5 out of 6 patients. There was an instant rise in the anti-SARS-CoV-2 antibody titers in 2 patients, but not all (Ye et al., 2020). These findings suggest that PC therapy is not only effective but also specific in COVID-19. In another CP evaluation study, 6 COVID-19 positive patients (tested by IgM ELISA) were given the therapy. After 11 days of CP transfusion, these patients did not need ventilator support (Zhang L. et al., 2020), which is indicative of the positive response of CP in COVID-19 treatment.

In another study, 5 severely ill patients with ARDS were given CP (two successive dosages of 200–250 ml) along with SARS-CoV-2-specific immunoglobulin G (IgG) titer. Four out of five patients showed body temperature reduction just after 3 days and an increase in the partial pressure of arterial oxygen and percentage of inspired oxygen ratio (PAO_2/FIO_2) after 12 days of treatment (Shen et al., 2020). Their sequential organ failure assessment score also reported being decreased along with the viral load, which became normal after 12 days of CP transfusion. After 14 days of the treatment, mechanical ventilation was not required, which is further indicative of the recovery of the COVID-19 patients. The investigational new drug (IND) regulatory steps led down by FDA to be followed by the investigators/licensed physicians to participate in the study who may have collected the CP from the COVID-19 recovered patients. CP seems to be an effective and safe therapy for critically ill patients infected with SARS-CoV-2. However, some clinical outcomes from multicentered randomized controlled trials are required to substantiate therapeutic promises of CP. A large number of clinical trials are going on

FOI-26-3132 Document 5

for assessment of therapeutic potency and safety of CP in COVID-19, and most of them are currently in phase 2 (NCT04359810, NCT04347681, NCT04345991, NCT04343261).

Conditional Support Therapy

Bronchodilators

Bronchodilators have the potential to enhance the risk of viral transmission with nebulized therapy. Therefore, there is not much role of bronchodilators in the supportive therapy and management of COVID-19 patients except there are chronic obstructive pulmonary disease or asthma (Institute for Safe Medication Practices, 2020). Even with such underlaying diseases, just the metered dose of inhalers is recommended.

NSAIDs

The role of nonsteroidal anti-inflammatory drugs (NSAIDs) in the management of COVID-19 patients is still under investigation (FDA, 2020). Drugs such as ibuprofen may increase angiotensin-converting enzyme 2 (ACE2) expression in the diabatic that could lead to detrimental results in COVID-19 patients. Ibuprofen may also increase the ACE2 expression in case of angiotensin II type-I receptor blockers treated patients (Loutfy et al., 2003; Fang et al., 2020). As a result, it is also suggested that the enhanced expression of ACE2 in the comorbid patients may facilitate the COVID-19 infection. Though, acetaminophen may be carefully used for fever control in the critically ill COVID-19 patients with high temperatures.

Corticosteroids

Corticosteroids (prednisone, methylprednisolone, dexamethasone, and budesonide) may be considered for patients with ARDS or refractory shock (Lian et al., 2020; Wu et al., 2020b; Zhou Y. H. et al., 2020). After exploring the published literature for corticosteroids against coronaviruses, we found that more studies are exploring the therapeutic promises of corticosteroids rather than NSAIDs (Lam et al., 2004; Wong et al., 2004; Chihrin et al., 2005). Overall, corticosteroids were found to have a positive response with respect to SARS-CoV due to their known capability to modulate the inflammations and reduce the immunopathological injury. One of the studies with porcine respiratory coronavirus infected pigs suggests that dexamethasone treatment with one or two doses of corticosteroids lessen early proinflammatory response. However, the prolonged use of corticosteroids may enhance the replication of virus (Zhang et al., 2008). On the contrary, a separate study with SARS-CoV patients indicated that early high dose of steroids with a quinolone gives the most favorable patient outcomes (Zhao et al., 2003). The studies on SARS-CoV infected mice model when treated with dexamethasone showed the alleviation of pulmonary inflammation (Hao et al., 2005).

Recent preliminary clinical trial results suggest that dexamethasone is effective in the life-saving of critically ill COVID-19 patients. This randomized, open-label controlled, adaptive, platform trial showed that dexamethasone reduced the mortality by one third and one fifth in patients getting invasive mechanical ventilation and oxygen support without mechanical ventilation, respectively. However, dexamethasone dose was not effective in reducing the mortality rate in patients not receiving any respiratory support (RECOVERY Collaborative Group, 2020). Results of the clinical trials for various corticosteroids, e.g., prednisone: NCT04344288, NCT04359511; methylprednisolone: NCT04343729; dexamethasone: NCT04360876; inhaled budesonide: NCT04355637 are waited for more clear evidence for the efficacy of corticosteroids in COVID-19.

Inhaled Pulmonary Vasodilators

The data about the use of pulmonary vasodilators are under investigation and aerosolized vasodilators to be avoided in the management of COVID-19 patients. There is no evidence to date about the efficacy of inhaled pulmonary vasodilators such as prostacyclins, nitric oxide, phosphodiesterase inhibitors, endothelin receptor antagonists, etc. in acute respiratory failures in SARS-CoV-2 infection (American Association for Respiratory Care, 2020; Department of Defense, 2020). Data regarding the clinical efficiency of nitric oxide are under investigation (NCT04305457, NCT04306393).

Anticoagulation

Low molecular weight heparin may be recommended to the COVID-19 patents, in the absence of contraindications such as platelet count < 25 x 109/L or active bleeding for venous thromboembolism prophylaxis. Therapeutic intensity anticoagulants may not be recommended for COVID-19 if venous thromboembolism is not present. During the heparin treatment with 449 COVID-19 patients, an increased D-dimer has been reported in the infected hospitalized patients. Mortality of the heparin treated patients was reported to be lower than non-treated patients after 28 days with SIC score ≥ 4 (Tang et al., 2020; Thachil et al., 2020). Ongoing clinical trials (NCT04345848, NCT04362085, NCT04359277, NCT04367831) data for therapeutic and prophylactic efficacy of anticoagulation in COVID-19 will establish better conclusions.

PROBABLE VACCINES

A total of 31 possible vaccines against COVID-19 have already reached to the clinical trials with 142 in the pre-clinical trials (**Table 1**). One such probable vaccine in the clinical trial stage is the recombinant adenovirus vector type 5-based vaccine, developed from the SARS-CoV-2 S gene using a non-replicating viral vector platform (Phase 1- ChiCTR2000030906; Phase 2- ChiCTR2000031781, NCT04313127). This study is sponsored by CanSino Biologics Inc. in Hubei, China and to be manufactured by Beijing Institute of Biotechnology, China. Phase 1 (n=108 healthy individuals) of the vaccine trial is an open, dose-escalating, and single-center study to assess the adverse reactions after 7 days of vaccine administration. Phase 2 (n= 500 healthy individuals) is randomized, placebo-controlled, and double-blind to assess the immunogenicity and safety by finding out the adverse reaction within 0-14 days, anti-SARS-CoV-2 antibody and SARS-CoV-2 neutralizing response after 28 days of the vaccine administration using intramuscular route.

FQI-26-3132 | Document 5

TABLE 1 | Probable vaccines under clinical trials for COVID-19 (as of August 28, 2020).

Vaccine Platform	Candidate Vaccine	Manufacturer/Developer	Route of Administration	
			Intramuscular	Intradermal
RNA	LNP-encapsulated mRNA	NIAID/Moderna	•	
	3 LNP-mRNAs	Pfizer/Fosun Pharma/BioNTech	•	
	mRNA	Curevac	•	
		Duke-NUS/Arcturus	•	
		Walvax Biotech/Academy of Military Sciences (PLA)	•	
	LNP-nCoVsaRNA	Imperial College, London	•	
Protein subunit	Adjuvanted recombinant protein (RBD-Dimer)	Chinese Academy of Sciences (Microbiology Institute)/Anhui Zhifei Longcom Biopharmaceutical	•	
	RBD-based	Kentucky Bioprocessing, Inc	•	
	Adjuvanted recombinant SARS CoV2 glycoprotein nanoparticle vaccine	Novavax	•	
	Trimeric subunit Spike protein (Native) vaccine	GSK/Dynavax/Clover Biopharmaceuticals	•	
	Advax™ adjuvant + Recombinant spike protein	Vaxine Pty Ltd/Medytox	•	
	Molecular clamp stabilized spike protein with MF59 adjuvant	CSL/Seqirus/University of Queensland	•	
	S-2P protein + CpG 1018	NIAID/Dynavax/Medigen Vaccine Biologics Corporation	•	
	RBD + Adjuvant	Instituto Finlay de Vacunas, Cuba	•	
	Peptide	FBRI SRC VB VECTOR, Rospotrebnadzor, Koltsovo	•	
Non-replicatingviral vector	Adenovirus type 5 vector	CanSino Biological Inc./Beijing Institute of Biotechnology	•	
	Ad26COVS1	Janssen Pharmaceuticals	•	
	Adeno-based	Gamaleya Research Institute	•	
	Replication defective Simian adenovirus (GRAd) encoding S	Univercells/ReiThera/LEUKOCARE	•	
	ChAdOx1-S	AstraZeneca/Oxford University	•	
Inactivated	Inactivated	Chinese Academy of Medical Sciences	•	
		Sinovac	•	
		Sinopharm/Wuhan Institute of Biological Products	•	
	Whole-Virion Inactivated	Bharat Biotech	•	
DNA	Plasmid vaccine with electroporation	International Vaccine Institute/Inovio Pharmaceuticals		•
	Plasmid vaccine + Adjuvant	AnGes/Takara Bio/Osaka University	•	
	Plasmid vaccine	Cadila Healthcare Limited		•
	Vaccine (GX-19)	Genexine Consortium	•	
Virus-like particles	Plant-derived VLP adjuvanted with GSK or Dynavax adjs.	GSK	•	
Replicating viral vector	Measles-vector based	Pittsburg University CVR/Merck Sharp & Dohme/Institute Pasteur/Themis	•	
		Sinopharm/Beijing Institute of Biological Products	•	

Here, • = Yes; Blank = No

Another vaccine at the clinical trial level is S gene-based synthetic vaccine, INO-4800 (NCT04336410). It is a DNA plasmid vaccine with electroporation for intradermal route of administration. This open-label study to evaluate the immunogenicity, tolerability, and safety profile of INO-4800 vaccine by testing two different dosages using CELLECTRA® 2000. It is currently in phase 1 (n=120) of the clinical trial with a time frame of 28 weeks, developed by Inovio Pharmaceuticals in Missouri and Pennsylvania, and manufactured by International Vaccine Institute, United States.

Two inactivated vaccine types are being evaluated for the safety and immunogenicity for SARS-CoV-2. Both are randomized, placebo-controlled, and double-blind evaluation studies. One sponsored by Sinovac Biotech Co., Ltd, at Jiangsu, China (NCT04352608) (n=744 estimated) is to evaluate the safety indices of adverse reactions (0–28 days), immunogenicity indexes of neutralizing-antibody (emergency vaccination schedule; 0–14 days, nonemergency vaccination schedule; 0–28 days) for 2 doses. The second is Vero cells based inactivated vaccine (ChiCTR2000031809), sponsored by Henan provincial center for disease control and prevention, China to be conducted in Beijing Institute of Biological Sciences/Wuhan Institute of Biological Sciences for immunogenicity and safety.

The fifth probable vaccine is mRNA-1273 (NCT04283461), which is under open-label dose-ranging trial (n=45 estimated) to evaluate its immunogenicity, reactogenicity, and safety upon twice 0.5 ml IM dose administration to deltoid muscle on 1st and 29th day. mRNA-1273 encodes for a full-length, prefusion stabilized S protein of SARS-CoV-2 and is a novel lipid nanoparticle (LNP)-encapsulated mRNA-based vaccine sponsored by ModernaTX, Inc, United States.

ChAdOx1 (NCT04324606) is another probable vaccine being developed using nonreplicating vector technology to be tested with IM administration. It is based on the adenovirus vaccine vector with SARS-CoV-2 spike protein. Developed by the University of Oxford, United Kingdom, and to be manufactured by AstraZeneca, aimed to conduct a randomized, single-blinded, multi-center phase 1 and 2 study (n= 1112 estimated) to assess

FOI 26-3132 Document 5

the immunogenicity, safety, and efficacy of the ChAdOx. It is single-dose probable vaccine.

There are some other notable probable vaccines are Anhui Zhifei Longcom Biopharmaceutical along and Institute of Microbiology, Chinese Academy of Sciences sponsored adjuvanted recombinant protein (RBD-Dimer), Osaka University, AnGes and Takara Bio sponsored DNA plasmid vaccine and adjuvant, Bharat Biotech's whole virion inactivated vaccine, Cadila's DNA plasmid vaccine, Arcturus/Duke-NUS sponsored mRNA vaccine, Genexine Consortium's GX-19 DNA vaccine, etc.

Apart from above mentioned probable vaccines, Codagenix and Serum Institute of India are investing in viral deoptimization to synthesize rationally designed live attenuated vaccine. It is in the pre-clinical stage and technical details of the proposed vaccine are awaited. To date, all these vaccine proposals are in the trial phase, and there is no approved vaccine specific for SARS-CoV-2 antiviral activity. In the pandemic paradigm, the development of a vaccine needs a quick start with many parallel steps without a pause as compared to the traditional vaccine development where linear steps with multiple analysis pauses are taken to reduce the attrition rate and financial losses. Therefore, it is imperative that some of these may fail; however, chances of getting a vaccine specific to SARS-CoV-2 seems to be higher due to the vast understanding of the viral genome at an unprecedented speed.

CONCLUSION

In the current outbreak, it is imperative to understand which patients might benefit from which set of therapy, especially when more than one disease progression pattern exists. The clinical data available so far is scant and inconsistent; therefore, a set of drugs or combinations or support therapy with complete therapeutic confidence is difficult to derive. Nevertheless, understanding of the treatment and management of COVID-19 patients is evolving very fast. The continuous emergence of clinical trial outcomes, pharmacologic therapy, and efficacy of available drugs for SARS-CoV-2 as clinical data is eagerly awaited. Investigation to assess the therapeutic promises of these drugs require a large number of controlled clinical trials and comparative evaluation for a better understanding of their effectiveness in COVID-19.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This work was funded by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University through the Fast-Track Research Funding Program. This work was supported by King Saud University, Deanship of Scientific Research, College of Science Research Center.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.582025/full#supplementary-material

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FOI 26-3132 Document 5

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Zinc supplementation for the prevention of pneumonia in children aged 2 months to 59 months (Review)

Lassi ZS, Moin A, Bhutta ZA

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Zinc supplementation for the prevention of pneumonia in children aged 2 months to 59 months.
Cochrane Database of Systematic Reviews 2016, Issue 12. Art. No.: CD005978.
DOI: 10.1002/14651858.CD005978.pub3.

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TABLE OF CONTENTS

HEADER 1
ABSTRACT 1
PLAIN LANGUAGE SUMMARY 2
SUMMARY OF FINDINGS 3
BACKGROUND 4
OBJECTIVES 4
METHODS 5
Figure 1. 6
RESULTS 7
Figure 2. 8
Figure 3. 9
DISCUSSION 10
AUTHORS' CONCLUSIONS 11
ACKNOWLEDGEMENTS 11
REFERENCES 12
CHARACTERISTICS OF STUDIES 15
DATA AND ANALYSES 24
Analysis 1.1. Comparison 1 Zinc supplementation vs placebo, Outcome 1 Pneumonia incidence. 25
Analysis 1.2. Comparison 1 Zinc supplementation vs placebo, Outcome 2 Pneumonia prevalence. 26
ADDITIONAL TABLES 26
APPENDICES 26
FEEDBACK 29
WHAT'S NEW 29
HISTORY 30
CONTRIBUTIONS OF AUTHORS 30
DECLARATIONS OF INTEREST 30
SOURCES OF SUPPORT 30
DIFFERENCES BETWEEN PROTOCOL AND REVIEW 30
INDEX TERMS 31

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[Intervention Review]

Zinc supplementation for the prevention of pneumonia in children aged 2 months to 59 months

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Editorial group: Cochrane Acute Respiratory Infections Group.
Publication status and date: Edited (no change to conclusions), published in Issue 1, 2017.

Citation: Lassi ZS, Moin A, Bhutta ZA. Zinc supplementation for the prevention of pneumonia in children aged 2 months to 59 months. *Cochrane Database of Systematic Reviews* 2016, Issue 12. Art. No.: CD005978. DOI: 10.1002/14651858.CD005978.pub3.

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ABSTRACT

Background

Pneumonia is a leading cause of morbidity and mortality in children younger than five years of age. Most deaths occur during infancy and in low-income countries. Daily zinc supplements have been reported to prevent acute lower respiratory tract infection (LRTI) and reduce child mortality. This is an update of a review first published in 2010.

Objectives

To evaluate the effectiveness of zinc supplementation in the prevention of pneumonia in children aged two to 59 months.

Search methods

We searched CENTRAL (Issue 21 October 2016), MEDLINE (1966 to October 2016), Embase (1974 to October 2016), LILACS (1982 to October 2016), CINAHL (1981 to October 2016), Web of Science (1985 to October 2016) and IMSEAR (1980 to October 2016).

Selection criteria

Randomised controlled trials (RCTs) evaluating zinc supplementation for the prevention of pneumonia in children aged from 2 months to 59 months.

Data collection and analysis

Two review authors independently assessed trial quality and extracted data.

Main results

We did not identify any new studies for inclusion in this update. We included six studies that involved 5193 participants.

Analysis showed that zinc supplementation reduced the incidence of pneumonia by 13% (fixed-effect risk ratio (RR) 0.87; 95% confidence interval (CI) 0.81 to 0.94, six studies, low-quality evidence) and prevalence of pneumonia by 41% (random-effects RR 0.59; 95% CI 0.35 to 0.99, one study, n = 609, low-quality evidence). On subgroup analysis, we found that zinc reduced the incidence of pneumonia defined by specific clinical criteria by 21% (i.e. confirmation by chest examination or chest radiograph) (fixed-effect RR 0.79; 95% CI 0.71 to 0.88, four studies, n = 3261), but had no effect on lower specificity pneumonia case definition (i.e. age-specific fast breathing with or without lower chest indrawing) (fixed-effect RR 0.95; 95% CI 0.86 to 1.06, four studies, n = 1932).

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Authors' conclusions

Zinc supplementation in children is associated with a reduction in the incidence and prevalence of pneumonia.

PLAIN LANGUAGE SUMMARY

Zinc supplementation for the prevention of pneumonia in children aged two to 59 months

Review question

We evaluated the effectiveness of zinc supplementation to prevent pneumonia in children aged from two to 59 months.

Background

Zinc is an essential element for children's growth and development. Too little zinc is associated with increased risk of infection, particularly diarrhoea and pneumonia. Children are more prone to zinc deficiency because they are less able to absorb dietary zinc and some children, especially in low-income countries, may not have received enough zinc from their mothers before birth. Zinc supplements for children has been reported to prevent pneumonia.

Search date

We searched the literature up to October 2016. This is an update of a review published in 2010. We did not find any new studies for this update.

Study characteristics

We included six studies that investigated zinc supplements to prevent pneumonia. The studies were conducted in Bangladesh, India, Peru and South Africa and involved 5193 children aged from two to 59 months. Children received either zinc or a similar-looking treatment that did not contain zinc. In two studies, children were also given vitamin A.

Study funding sources

All included studies were funded. Of these, three explicitly mentioned that funding agencies had no role in the design and results of the study.

Key results

Zinc supplementation was significantly associated with reducing the incidence and prevalence of pneumonia among children aged from two to 59 months. On subgroup analysis, we found that a more stringent diagnosis (radiological examination) increased the reduction in pneumonia incidence.

Quality of the evidence

Overall, evidence quality was assessed as low on GRADE assessment.

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SUMMARY OF FINDINGS

Summary of findings for the main comparison. Zinc supplementation compared with placebo for the prevention of pneumonia in children aged 2 months to 59 months

Zinc supplementation compared with placebo for the prevention of pneumonia in children aged 2 months to 59 months

Patient or population: children aged 2 months to 59 months
Settings: Bangladesh, India, Peru, South Africa
Intervention: zinc supplementation
Comparison: placebo

Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	No of Participants (studies)	Quality of the evidence (GRADE)	Comments
	Assumed risk	Corresponding risk				
	Placebo	Zinc supplementation				
Pneumonia incidence	**343 per 1000**	**299 per 1000** (278 to 323)	**RR 0.87** (0.81 to 0.94)	5193 (6 studies)	⊕⊕⊖⊖ **low** [1,2]	
Pneumonia prevalence	**22 per 1000**	**13 per 1000** (8 to 22)	**RR 0.59** (0.35 to 0.99)	609 (1 study)	⊕⊕⊖⊖ **low** [3,4]	

*The basis for the **assumed risk** (e.g. the median control group risk across studies) is provided in footnotes. The **corresponding risk** (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).
CI: Confidence interval; **RR:** Risk ratio;

GRADE Working Group grades of evidence
High quality: Further research is very unlikely to change our confidence in the estimate of effect.
Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.
Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.
Very low quality: We are very uncertain about the estimate.

[1] Studies have unclear information on allocation concealment, blinding and reporting biases.
[2] Pneumonia diagnosis criteria was used differently.
[3] Studies have limited information on blinding of outcomes assessors and reporting bias.
[4] Total number of events were less than 300.

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BACKGROUND

Description of the condition

Pneumonia is the largest cause of childhood mortality, accounting for 15% of all childhood deaths under five years (WHO 2015), and 19% of all childhood deaths in low-income countries (Bryce 2005; Rudan 2008). Pneumonia is diagnosed by the presence of either fast breathing or lower chest wall indrawing (WHO 2015) in children aged under five years. Presenting features in viral or bacterial pneumonia are similar, however wheezing is more common in children with viral pneumonia.

Pneumonia can be prevented by immunising against *Haemophilus influenzae* type B (Hib), pneumococcus, measles and pertussis (whooping cough) (WHO 2015). Nutrition including breastfeeding for the first six months of life plays a major role by boosting immunity against causative organisms of pneumonia (WHO 2015). Good hygiene and clean indoor air also help in preventing pneumonia (WHO 2015).

Rudan 2013 identified five risk factors for pneumonia: malnutrition, low birth weight, nonexclusive breast feeding, solid fuel use and overcrowding. By 2010, pneumonia episodes for children aged under four years in low- and middle-income countries (LMICs) decreased by 25% over the past decade to about 0.22 episodes per child per year when exposure to the five risk factors decreased by 20% to 30% (Rudan 2013). Therefore, any interventions that can improve child survival from pneumonia are important.

According to the World Health Organization (WHO), zinc deficiency accounts for 13% of all lower respiratory tract infection (LRTIs) (WHO 2009). Acute LRTI is a leading cause of mortality among children aged under five years (Bryce 2005; Rudan 2008), and which is responsible for nearly two million deaths annually, mainly in low-income countries.

Description of the intervention

In 2005, zinc intake was inadequate among 17.3% of the world's population; rates of zinc adequacy are lower in sub-Saharan Africa and South Asia (Wessells 2012). The World Bank described multiple factors such as low animal food intake, less bioavailability of dietary zinc, and zinc loss during events of diarrhoea (Bhutta 1999; Black 1998) that could potentially lead to zinc deficiency in low-income settings (World Bank 2016). The WHO estimated that zinc deficiency may be related to 800,000 deaths a year, with more than 50% occurring in children under five years of age. Children are more susceptible to zinc deficiency because of lower rates of intestinal absorption and from starting life with low body stores due to intrapartum under-nutrition (Krebs 2014).

Mild-to-moderate zinc deficiency is associated with impaired physical growth, delayed sexual maturity, behavioural disturbances, anorexia, affected permeability of the intestinal tract and decreased immunocompetence as well as subclinical inflammation; moderate-to-severe deficiency can lead to diarrhoea; and severe deficiency produces dermatitis and alopecia (Krebs 2014).

There is evidence that preventative zinc supplementation reduces pneumonia morbidity (Bhutta 1999; Yakoob 2011) and is beneficial in reducing diarrhoeal episodes in children with acute and persistent diarrhoea (Bhandari 1994; Zinc Group 2000). However, there is insufficient evidence to support preventive zinc supplementation or adjunctive zinc supplementation with antibiotics to reduce pneumonia-specific mortality (Haider 2011; Yakoob 2011). Zinc supplements (zinc sulphate, zinc gluconate, zinc acetate) can be administered as tablets, syrup or powder. However, zinc supplements need to be administered with caution because excess levels may cause toxicity.

How the intervention might work

Zinc plays an important role in cell regeneration, immunity and growth (Krebs 2014; Shankar 1998). Zinc deficiency decreases T-lymphocytes and T-helper, impairs macrophage function and reduced killer cells (Ibs 2003; Ravaglia 2000), and adversely impacts innate immunity affecting interferon (IFN) gamma production, interleukin-2 (IL-2) and tumour necrosis factor-α (TNF-α) (Krebs 2014). Zinc supplementation in children increases levels of complement in the blood that modulate the function of T-lymphocytes, T-helper, macrophages and neutrophils and hence improves the ability to fight infection. Zinc supplementation improves circulating levels of T-lymphocytes and other macrophages that enhance ability to fight infection (Fraker 1993). Zinc is not stored in the body, but repletion is straightforward. Children with diarrhoea can become zinc deficient quickly. Furthermore, if zinc deficiency occurs during periods of growth, it can be conducive to growth faltering as is shown by a review that found correlation between dietary zinc inadequacy and stunting among children aged under four years (Krebs 2014; Wessells 2012).

Why it is important to do this review

Bhutta 1999 showed that zinc was associated with a reduced incidence of pneumonia. Aggarwal 2007 included more recent studies, but included children aged between birth and 59 months who were given zinc supplements for at least a few months. Roth 2010 calculated the effect size by case definition of pneumonia, and included studies that administered zinc supplements to children aged between birth and 59 months. Yakoob 2011 focused on zinc supplementation for more than three months in children aged under five years and found evidence to support the positive impact of zinc supplementation on diarrhoeal and pneumonia morbidity and mortality to include it in the LiST tool. However Yakoob 2011 did not include low birth weight or small-for-gestational age infants, nor provide a specific case definition for pneumonia. Similarly, a review by Bhutta 2013 of diarrhoea and pneumonia interventions reported a non-significant impact of preventive zinc supplementation on acute lower respiratory infection (ALRI)-related mortality.

We investigated zinc supplementation for at least three months in children aged from two months to 59 months and conducted a meta-analysis of data published up to 2011. However, with new and upcoming trials, this review required updating.

OBJECTIVES

To evaluate the effectiveness of zinc supplementation in the prevention of pneumonia in children aged two to 59 months.

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METHODS

Criteria for considering studies for this review

Types of studies

We included randomised controlled trials (RCTs) evaluating supplementation of zinc for the prevention of pneumonia in children aged from two to 59 months. We included studies that defined an episode of pneumonia in the following ways.

1. Reported cough or difficulty in breathing with a respiratory rate above the WHO-defined age-specific values (respiratory rate of 50 breaths per minute or more for children aged two to 11 months, or respiratory rate of 40 breaths per minute or more for children aged 12 to 59 months), and either documented fever over 38°C or chest in-drawing (WHO 1990).
2. A diagnosis of pneumonia based on chest examination by a physician.
3. A diagnosis of pneumonia based on a chest radiograph.

We included trials published in languages other than English. Quasi-RCTs were not eligible for inclusion.

Types of participants

We included children aged from two months to 59 months.

Types of interventions

Oral supplement containing at least the USA's recommended daily allowance of zinc versus either an oral supplement without zinc or placebo. We excluded trials in which children were given additional supplements unless the co-interventions other than zinc were the same in both groups. The recommended daily allowance for infants is 5 mg of elemental zinc per day and 10 mg per day for children aged from one to five years (RDA 1989). We included trials in which supplements were administered for at least three months and outcome surveillance was carried out for at least four weeks.

Types of outcome measures

Primary outcomes

1. Numbers of new episodes of pneumonia in children aged from two months to 59 months.

Secondary outcomes

1. Prevalence (number of cases of pneumonia at a given time per total days of observation) of pneumonia in children aged from two months to 59 months.

Search methods for identification of studies

Electronic searches

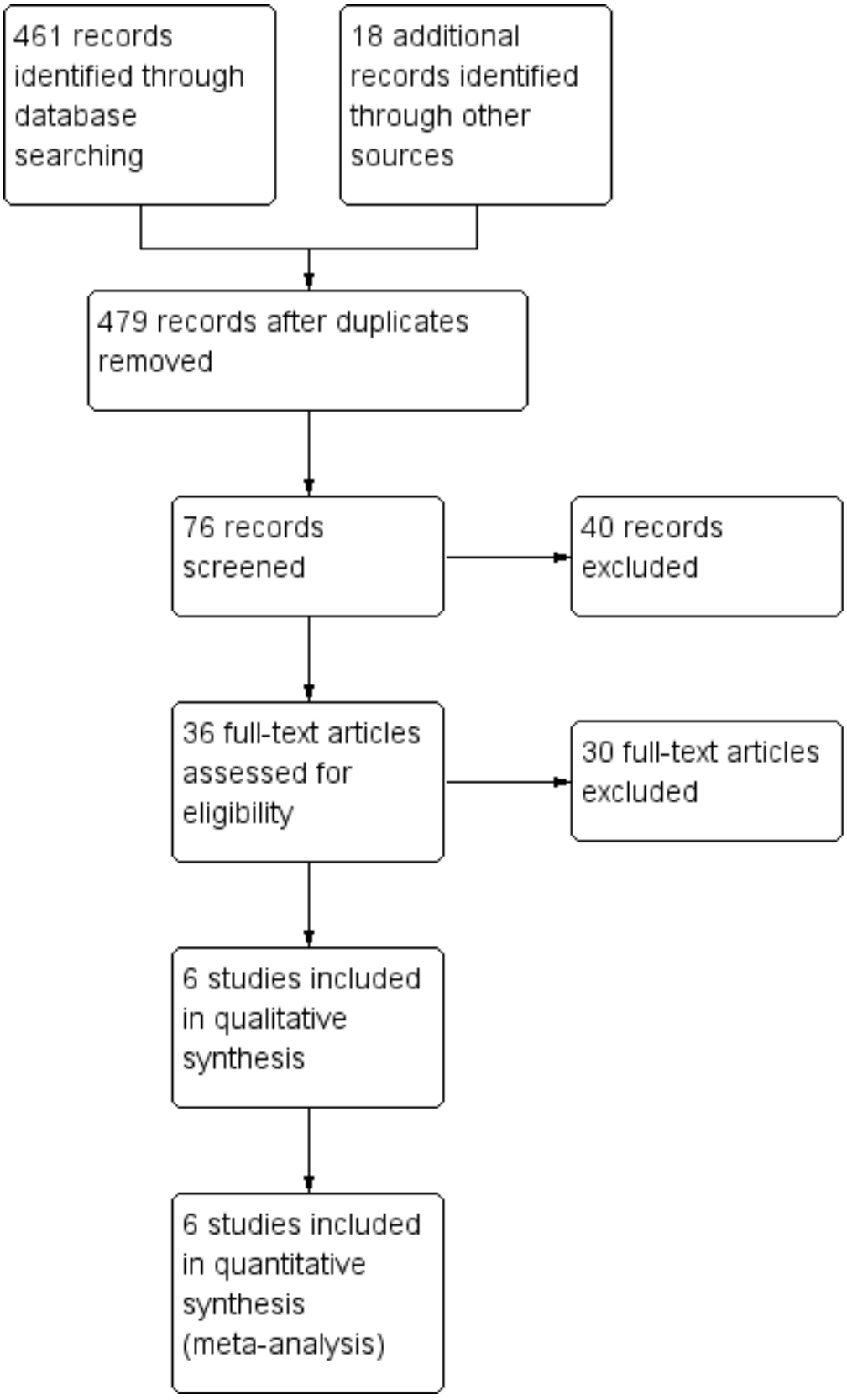
We searched the Cochrane Central Register of Controlled Trials (CENTRAL), the Cochrane Library (www.thecochranelibrary.com, accessed 21 October 2016), which contains the Acute Respiratory Infections Group's Specialised Register, MEDLINE (November 2009 to 21 October 2016) and Embase (January 2010 to 21 October 2016). We searched LILACS (1982 to 21 October 2016) using a new search strategy. We also searched CINAHL (1981 to 21 October 2016) and Web of Science (1985 to 21 October 2016). Details of previous searches are presented in Appendix 1.

We used the search strategy described in Appendix 2 to search MEDLINE and CENTRAL. We combined the MEDLINE search with the Cochrane Highly Sensitive Search Strategy for identifying randomised trials in MEDLINE: sensitivity- and precision-maximising version (2008 revision); Ovid format (Lefebvre 2011) . We adapted the search strategy to search Embase (Appendix 3), LILACS (Virtual Health Library) (Appendix 4), CINAHL (Appendix 5) and Web of Science (Appendix 6). We searched IMSEAR using the search terms "pneumonia" and "zinc". We imposed no language or publication restrictions.

Figure 1 illustrates the search and study selection processes.

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Figure 1. Study flow diagram



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Searching other resources

We searched the WHO ICTRP (www.who.int/ictrp) and ClinicalTrials.gov trials registries (latest search 8 July 2016). We also searched related conference proceedings for relevant abstracts. We also checked the reference lists of all trials identified.

Data collection and analysis

Selection of studies

Two review authors independently assessed all potential studies identified for inclusion. We resolved any disagreement through discussion, and if required, consulted the third review author.

Data extraction and management

We designed a data extraction form. Two review authors extracted data. We resolved discrepancies through discussion, and if required, we consulted the third review author. We entered data into Review Manager software (RevMan 2014) and checked for accuracy. There were no new studies identified for inclusion in this update.

Assessment of risk of bias in included studies

There were no new studies identified for inclusion for this update. For the 2010 version of this review (Lassi 2010), two review authors (ZSL, BAH) independently assessed risk of bias using the criteria outlined in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011). We resolved any disagreement by discussion with the third review author (ZAB).

Measures of treatment effect

For dichotomous outcomes, we extracted the total number of participants for each group and numbers of participants experiencing an event. We used risk ratio (RR) and 95% confidence intervals (CIs) to describe effect sizes.

Unit of analysis issues

We carried out statistical analysis using RevMan 2014). Initially, we used fixed-effect inverse variance meta-analysis to combine data where trials examined the same intervention, and study populations and methods were judged sufficiently similar. Where we suspected clinical or methodological heterogeneity between studies sufficient to suggest that treatment effects may differ between trials, we conducted random-effects meta-analysis. When we identified substantial heterogeneity in a fixed-effect meta-analysis, we noted this and repeated the analysis using the random-effects method.

Dealing with missing data

We noted levels of attrition among included studies. As far as possible, we performed analyses on an intention-to-treat (ITT) basis. We attempted to include all participants randomised to each group in the analyses.

Assessment of heterogeneity

We measured heterogeneity among the trials by calculating the I^2 statistic, and Chi^2 P value. If the I^2 statistic exceeded 50%, and the Chi^2 P value was less than 0.1, we would have considered heterogeneity to be substantial. We did not find any heterogeneity; therefore, we did not attempt subgroup analyses based on differences in zinc dosage, participants' age, supplementation in healthy children compared with children recovering from an episode of illness, and pre-intervention zinc levels. However, we attempted to look for the differential effect of zinc supplementation on the case definition of ALRI.

Assessment of reporting biases

We aimed to assess reporting bias by comparing published study reports with study protocols. We planned to assess for the presence of publication bias by looking for funnel plot asymmetry; however, the low number of included studies meant this was not feasible.

Data synthesis

We carried out statistical analysis using RevMan 2014 software. In the absence of significant heterogeneity, we used the fixed-effect meta-analysis model for combining data.

Subgroup analysis and investigation of heterogeneity

We planned to perform subgroup analysis based on diagnosis of pneumonia.

Sensitivity analysis

We planned to carry out a sensitivity analysis to explore the effect of trial quality (assessed by concealment of allocation), by excluding studies with clearly inadequate allocation concealment. However, only two studies had adequate allocation concealment, therefore we did not run the sensitivity analysis.

RESULTS

Description of studies

See Characteristics of included studies, Table 1 and Characteristics of excluded studies tables.

Results of the search

This is an update of a review previously published in 2010 (Lassi 2010). The most recent search (to October 2016) did not identify any new studies for inclusion.

Included studies

We included six trials. These studies were from Bangladesh (Brooks 2005), India (Bhandari 2002; Sazawal 1998), Peru (Penny 2004), and South Africa (Bobat 2005; Luabeya 2007).

Penny 2004 conducted a randomised, double-blind, placebo-controlled community-based trial in Lima, Peru that involved 238 children with diarrhoea for more than 14 days who were aged from six to 36 months. Children were randomised to receive zinc alone, zinc plus vitamins or placebo for two weeks. Childrens' characteristics at baseline were similar except for sex and length-for-age Z scores.

Brooks 2005 conducted a study in a low-income population in an urban setting of Dhaka, Bangladesh. The study included 1665 children aged from 60 days to 12 months who were randomly allocated to receive oral zinc (70 mg) or placebo once weekly. Children were assessed weekly in the children's homes. Children with suggestive respiratory disease or diarrhoea were referred to clinics where they were assessed and diagnoses made based on the WHO criteria.

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Bhandari 2002 included 2482 children aged six to 30 months who were from urban slums in New Delhi, India. Children received daily elemental zinc (10 mg for infants; 20 mg for older children) or placebo for four months. Both groups received single dose vitamin A (100,000 IU for infants and 200,000 IU for older children) at enrolment. Both groups were comparable on socio-demographic profile, anthropometry, child feeding practices, morbidity in the previous 24 hours, and plasma zinc concentration.

Luabeya 2007 included children aged from four to six months from the KwaZulu-Natal Province of South Africa. Children were randomised to two intervention arms and one control arm. Children in the control arm received vitamin A only; in the first intervention arm they were given vitamin A plus zinc; and in the second intervention arm children received vitamin A, zinc and multiple micronutrients. Children received supplements until 24 months of age, thus each child received zinc supplements for 18 to 20 months. Among the participants, 32 children were born with HIV positive status, and 154 were born without HIV infection to HIV positive mothers. Groups differed on weight-for-length scores.

The double-blind controlled trial by Sazawal 1998 was conducted in a low socioeconomic population of 609 children aged between six and 35 months in urban India. Children were assigned to zinc supplementation (n = 298) and placebo (n = 311) groups. Children in the treatment group received 10 mg elemental zinc and placebo group children received a substance similar in colour and taste. The daily fixed dose of 5 mL per child was given to all enrolled children for six months, but this was increased to 10 mL for children with diarrhoeal illnesses. The baseline characteristics of included children were similar for age, sex, nutritional status and baseline plasma zinc.

Bobat 2005 conducted a randomised, double-blind, placebo-controlled equivalence trial of zinc supplementation at Grey's Hospital in Pietermaritzburg, South Africa. The study included 96 children aged from six to 60 months with HIV 1 infection who were randomly assigned to receive 10 mg of elemental zinc sulphate or placebo daily for six months. Baseline characteristics of included children were similar.

Excluded studies

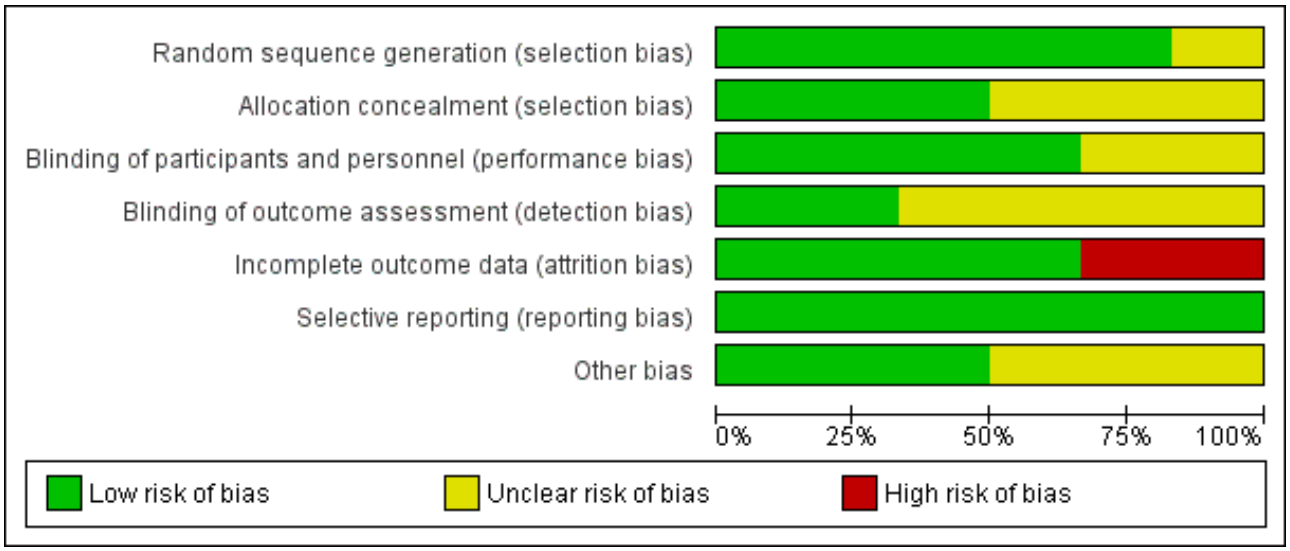
We excluded 30 studies that did not satisfy inclusion criteria. Seven studies included children outside the age limits of our review criteria (Lira 1998; McDonald 2015; Osendarp 2002; Sur 2003; Taneja 2009; Tielsch 2007 (also reported a different response to intervention definition than we used); Vakili 2009). We excluded seven studies that provided supplements for less than three months (Baqui 2002; Castillo-Duran 1987; Chandyo 2010; Feiken 2014; Rahman 2001; Roy 1999; Sempértegui 1996). We excluded 14 studies that applied different case definitions for ALRI/pneumonia, which were not based on WHO criteria, radiograph or chest examination by physician (Baqui 2003; Larson 2010; Long 2006; Lind 2004; Malik 2014; Mazoomar 2010; Ninh 1996; Reul 1997; Richard 2006; Sampaio 2013; Sanchez 2014; Soofi 2013; Rosado 1997; Umeta 2000). We excluded one study because children received zinc supplement in a fortified drink (Bates 1993). We also excluded one study (Adhikari 2016) because it had included children with recurrent infections.

Please refer to Characteristics of excluded studies for further details.

Risk of bias in included studies

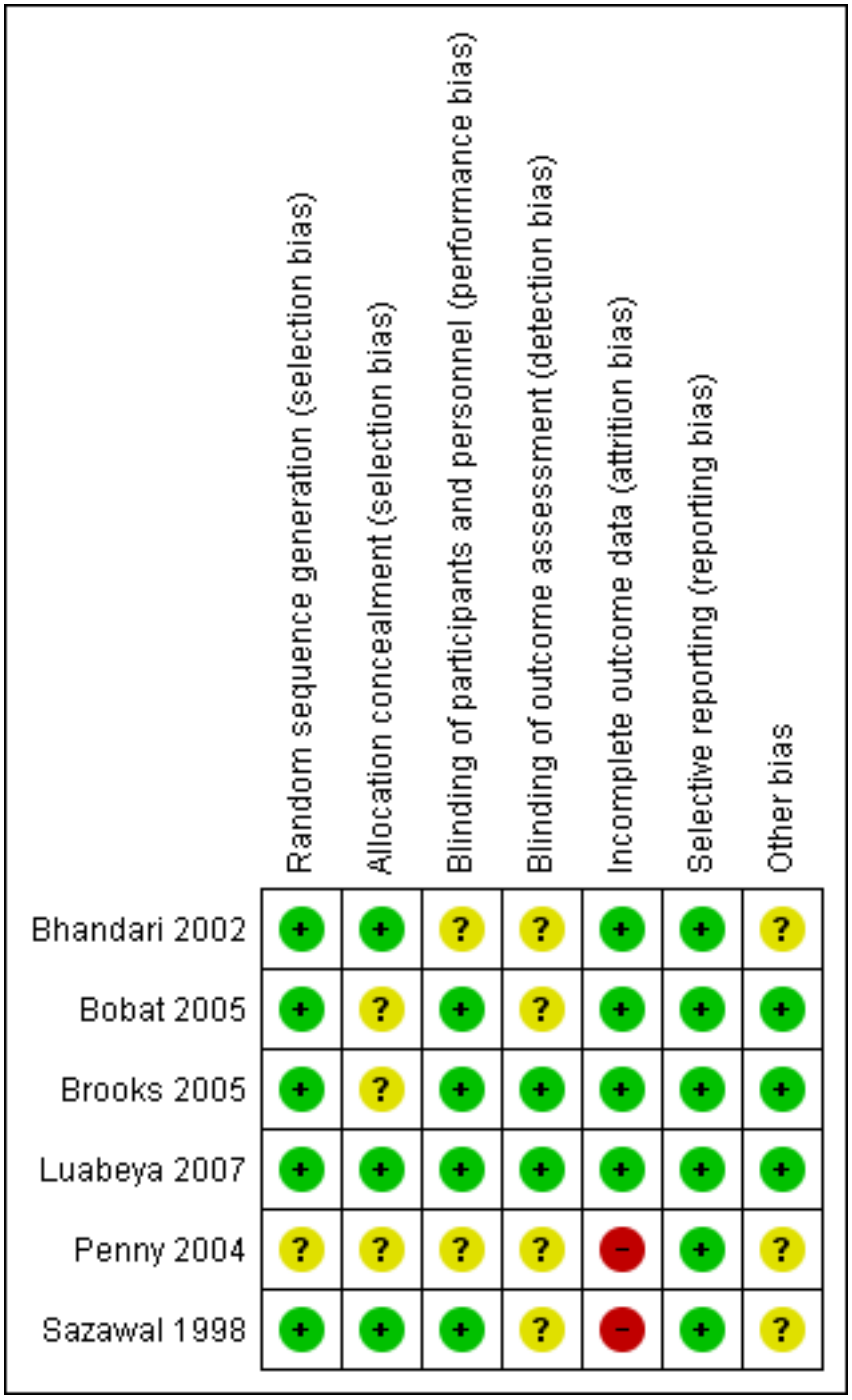
Figure 2 and Figure 3 provide graphical summaries of the 'Risk of bias' assessment for the six included studies. With one exception (Penny 2004), studies were assessed as providing low or unclear risk of bias in relation to methods. Luabeya 2007 had the lowest risk of bias; Penny 2004 was assessed at unclear risk of bias.

Figure 2. Methodological quality graph: review authors' judgements about each methodological quality item presented as percentages across all included studies



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Figure 3. Methodological quality summary: review authors' judgements about each methodological quality item for each included study



Allocation

Most included studies were assessed as demonstrating adequate sequence generation and used computer-generated sequencing techniques (Bhandari 2002; Bobat 2005; Brooks 2005; Luabeya 2007; Sazawal 1998). Allocation concealment was adequate three studies (Bhandari 2002; Luabeya 2007; Sazawal 1998); the remainder did not report in sufficient detail to enable assessment (Bobat 2005; Brooks 2005; Penny 2004).

Blinding

Blinding of participants and study personnel was achieved in four studies (Bobat 2005; Brooks 2005; Luabeya 2007; Sazawal 1998). The remaining studies reported insufficient information to permit judgement (Bhandari 2002; Penny 2004).

Blinding of outcome assessors was achieved in Brooks 2005 and Luabeya 2007. Brooks 2005 reported that blinding was not affected because a proportion of children in both the zinc and placebo

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groups reacted to the taste such that treatment could not be distinguished; Luabeya 2007 reported that outcomes assessors were blinded to assignment.

Incomplete outcome data

Attrition and exclusions were described in all except studies Penny 2004 and Sazawal 1998. The other four studies (Bhandari 2002; Bobat 2005; Brooks 2005; Luabeya 2007) reported reasons for exclusions; these included refusal to participate, moved outside the study site, taste of the syrup, and death.

Selective reporting

Only Luabeya 2007 was registered with a trials register. None of the other studies had published protocols (Bhandari 2002; Bobat 2005; Brooks 2005; Penny 2004; Sazawal 1998). However, all proposed outcomes were reported.

Other potential sources of bias

The funding agencies were reported to have had no input to design or study results in three studies (Bobat 2005; Brooks 2005; Luabeya 2007). Bhandari 2002; Penny 2004 and Sazawal 1998 did not mention the role of funding agencies explicitly.

Effects of interventions

See: **Summary of findings for the main comparison** Zinc supplementation compared with placebo for the prevention of pneumonia in children aged 2 months to 59 months

All six studies reported the incidence of pneumonia (Bhandari 2002; Bobat 2005; Brooks 2005; Luabeya 2007; Penny 2004; Sazawal 1998), while one study (Sazawal 1998) also reported prevalence rates.

Incidence of pneumonia

Administration of zinc supplementation showed a statistically significant impact on reducing pneumonia incidence by 13% (risk ratio (RR) 0.87, 95% confidence interval (CI) 0.81 to 0.94, fixed-effect model, six studies, n = 5193, low-quality evidence). There was no heterogeneity (I^2 statistic = 28%, Chi^2 test P = 0.20; Analysis 1.1).

We pooled studies that used similar case definitions. Studies that used clinical definitions of age-specific fast-breathing, with or without lower chest indrawing did not exhibit impact from zinc supplementation on reducing pneumonia (RR 0.95, 95% CI 0.86 to 1.06, fixed-effect model, four studies, n = 1932). There was no heterogeneity (I^2 statistic = 0%, Chi^2 test P = 0.74).

Studies that applied case definition for pneumonia based on chest examination or chest radiograph exhibited significant impact from zinc supplementation on reducing incidence of pneumonia by 21% (RR 0.79, 95% CI 0.71 to 0.88, fixed-effect model, four studies, n = 3261). There was no heterogeneity (I^2 statistic = 0%, Chi^2 test P = 0.44). Bobat 2005 included children with HIV. When this study was removed from the analysis there was no difference in results (RR 0.88, 95% CI 0.81 to 0.95, fixed-effect model, three studies, n = 3165). Evidence was low quality (Summary of findings for the main comparison).

Prevalence of pneumonia

Administration of zinc supplementation showed a statistically significant impact on reducing the pneumonia prevalence by 41% among children aged from two to 59 months (RR 0.59, 95% CI 0.35 to 0.99, fixed-effect model, one study, n = 609, low-quality evidence; Analysis 1.2). (Summary of findings for the main comparison).

DISCUSSION

Summary of main results

This is an update of a review first published in 2010 and included no new studies. Previous conclusions are unchanged.

We included six randomised controlled trials (RCTs) evaluating the impact of zinc supplementation in children aged from two to 59 months. Our meta-analysis indicated that zinc supplementation led to reductions in the incidence of pneumonia by 13% and pneumonia prevalence by 41%. The greater overall reduction of pneumonia prevalence result is due to prevalence data originating from one study.

We found that zinc reduced pneumonia incidence (defined by specific clinical criteria and confirmed by chest examination or chest radiograph) by 21%. We found an expected association of acute lower respiratory infection (ALRI) case definition with an effect size (P = 0.02). We found benefits of zinc supplementation when ALRI was diagnosed following clinical examination or chest radiography suggesting infection.

Reduction in pneumonia incidence supports the use of zinc supplements among children two to 59 months of age. Because zinc is not stored in the body, adequate zinc needs to be available in daily diet (Sanstead 1995). Children, particularly those from low-income countries who have inadequate intake of food that contains zinc (mainly foods of animal origin) should receive supplements to address deficiency.

Overall completeness and applicability of evidence

Our findings from can be extrapolated for children living in low- and middle-income countries because most included studies were from disadvantaged urban areas of low-income countries (Bangladesh, India, Peru, and South Africa). In two studies, either children or their mothers had HIV positive status (Bobat 2005; Luabeya 2007). In most studies, children were excluded if they had co-morbidities such as tuberculosis, congenital heart disease, less than 60% median weight-for-age Z score, and nutritional oedema. Penny 2004 recruited children on their breastfeeding status. The use of consistent and accurate definitions of pneumonia, with emphasis on clinical documentation of key signs or radiographic diagnosis, would avoid misclassification and lead to greater confidence in study findings.

Quality of the evidence

We assessed that review outcomes were low according to GRADE criteria. We downgraded evidence quality because outcomes were ascertained using different criteria and 'Risk of bias' assessment. Of note, half of the included studies reported pneumonia according to the WHO definition (WHO 1990); the remainder relied on clinical examination and chest radiographs. We therefore looked for differential effects on outcome estimates with respect to case definition and reported their impact separately.

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Allocation concealment was adequately described overall. All included studies were deemed to be adequately blinded for treatment assignment. Completion rates were greater than 90% in four included studies; Penny 2004 reported completion by 16% of participants and Sazawal 1998 did not report numbers of participants who dropped out. The low levels of missing data meant that we chose not to assess the impact on overall estimates because it was not anticipated to cause significant bias in the study results.

Potential biases in the review process

We undertook a systematic search of the literature to identify all studies that met our inclusion criteria. Study selection and data extraction decisions were cross-checked independently by the review authors. Included studies were not free from bias; only one study was assessed at low risk of bias for all domains. We could not assess reporting bias because of the limited numbers of included studies.

Agreements and disagreements with other studies or reviews

We found RCT evidence from both high- and low-income countries showing an effect of zinc in decreasing morbidity and mortality in children due to respiratory and gastro-intestinal infections (Hambidge 1999; Sazawal 1998). The effect of zinc against infectious diseases is therapeutic as well as preventive.

Findings reported by the Zinc Investigators' Collaborative Group in (Bhutta 1999) and Aggarwal 2007 were consistent with our review. Bhutta 1999 reported that children who received zinc supplements had positive effects for pneumonia (odds ratio (OR) 0.59, 95% CI 0.41 to 0.83). Moreover, Bhutta 1999 included trials that administered zinc supplements with therapeutic intent, which might have led to an overestimation of the potential preventive effects of zinc. Aggarwal 2007 included trials that recruited children aged from birth to 59 months who were provided zinc supplementation for at least three months; analysis showed 20% decreased incidence of respiratory illness among children supplemented with zinc compared with placebo. Similar findings were reported by Roth 2010 who also assessed specific case definitions for impact evaluation of children aged from birth to five years. Roth 2010 reported that zinc reduced ALRI incidence defined by specific clinical criteria (incidence rate ratio (IRR) 0.65, 95% CI 0.52 to 0.82), compared with no effect on lower-specificity ALRI case definitions based on caregiver reports (IRR 1.01, 95% CI 0.91 to 1.12) or WHO non-severe pneumonia (IRR 0.96, 95% CI 0.86 to 1.08).

AUTHORS' CONCLUSIONS

Implications for practice

We found sufficient evidence to support the use of zinc supplementation for children aged from two to 59 months to prevent pneumonia. Our analysis supports use of preventive zinc supplementation to improve child health.

Implications for research

More well-designed, large-scale randomised controlled trials (RCTs) are needed to establish the benefit of zinc supplementation for preventing pneumonia among children aged from two to 59 months. Development of effective and feasible interventions to improve zinc status in children is essential. Enhancement of bioavailable zinc in foods by genetic engineering, plant breeding and periodical supplementation are other possible intervention strategies that should be evaluated.

Given the rising burden of child mortality due to respiratory infections, particularly pneumonia, and considering its decreasing impact with zinc supplementation, further reviews should be considered in which the effectiveness of zinc supplementation should be assessed for acute pneumonia provided that cases are well-defined by strict clinical criteria.

ACKNOWLEDGEMENTS

The authors wish to thank Ammad Saeed, who assisted in the development of protocol and Ms Sarah Thorning for her assistance with the literature search. The review authors would like to thank the following people for commenting on the draft review: Abdullah Brooks, Robert Black, Teenah Handiside and Jiaan-Der Wang, Mark Griffin, and John Holden.The draft protocol was written by Dr Batool A Haider (BAH) who also took part in the initial stages of the 2010 review and assisted in data extraction.

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* Indicates the major publication for the study

CHARACTERISTICS OF STUDIES

Characteristics of included studies *[ordered by study ID]*

Bhandari 2002

Methods	RCT in which the children were individually randomised by a computer-generated simple randomisation scheme in blocks of 8. Zinc or placebo bottles were labelled with a unique child identification number according to the randomisation scheme. Six bottles, one for each month and two extra, for each child were produced and labelled before enrolment commenced. The supplies for each child were kept separately in labelled plastic bags. The zinc and placebo syrups were similar in appearance, taste, and packaging. Blinding was maintained during analyses by coding the groups as A or B.

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Bhandari 2002 *(Continued)*

	The study took place in the urban slum of Dakshinpuri in New Delhi, India. For episodes to be counted as individual, there had to be at least 14 intervening days. The children in the two groups were comparable for age, anthropometry, child feeding practices, morbidity in the previous 24 hours, socioeconomic characteristics and plasma zinc concentration.
Participants	The study included children aged from 6 to 30 months. There were 1241 children in each group, and after dropouts, the number reduced to 1093 in the zinc and 1133 in the placebo groups. Children were excluded if consent was refused, were likely to move out of the study area within the next four months, needed urgent admission to hospital on the enrolment day or had received a massive dose of vitamin A (100,000 IU for infants and 200,000 IU for older children) within the two months before enrolment.
Interventions	Doses of elemental zinc were 10 mg for infants and 20 mg for older children (twice the recommended daily dosage) as zinc gluconate. Zinc or placebo was taken daily for four months. Both groups received single massive doses of vitamin A (100,000 IU for infants and 200,000 IU for older children) at enrolment. Immunisations and treatment for acute illnesses were provided as per WHO guidelines. Children with acute lower respiratory tract infections received cotrimoxazole. Amoxicillin was substituted if the child did not respond within three days. Children were sent to hospital if they had signs and symptoms that warranted referral according to WHO guidelines.
Outcomes	Incidence of ALRI ALRIs were defined by cough and fast breathing or lower chest indrawing as assessed by the physician; other clinical signs were not taken into account. Fast breathing was defined as 2 counts of > 50 breaths/minute for infants and > 40 breaths/minute for older children. Pneumonia was diagnosed either by a combination of cough with crepitations or bronchial breathing by auscultation or as an episode of ALRI associated with at least one of lower chest indrawing, convulsions, not able to drink or feed, extreme lethargy, restlessness or irritability, nasal flaring, or abnormal sleepiness.
Notes	Funding: European Union (Contract No IC18CT960045), Norwegian Council of Universities' Committee for Development Research and Education (PRO 53/96), Department of Child and Adolescent Health and Development (CAH), WHO.

Risk of bias

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "children were individually randomised by a simple randomisation scheme in blocks of eight. The randomisation scheme was generated by a statistician at Statens Serum Institut, not otherwise involved with this study, using the SAS software"
Allocation concealment (selection bias)	Low risk	Quote: "zinc or placebo syrups were prepared and packaged in unbreakable bottles by GK Pharma ApS Koge, Denmark, who also labelled bottles with a unique child identification number according to the randomisation scheme. The supplies of each child were kept separately in labelled plastic bags. The zinc and placebo were similar in appearances, taste and packaging. Masking was maintained during the analysis by coding the groups as A and B"
Blinding of participants and personnel (performance bias) All outcomes	Unclear risk	Quote: "the supplies for each child were kept separately in labelled plastic bags". "Masking was maintained during analyses by coding the groups as A or B"
Blinding of outcome assessment (detection bias) All outcomes	Unclear risk	Insufficient information to permit judgment

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Bhandari 2002 *(Continued)*

Incomplete outcome data (attrition bias) All outcomes	Low risk	Exclusion (35%) with their reasons documented. Attrition was 12% in the zinc group and 8.7% in the control group. Loss to follow-ups were mainly because they refused further participation, moved and died (3 died in the placebo group only)
Selective reporting (reporting bias)	Low risk	We could not locate the protocol of this study. We could not find the trial registration number of the study. The outcomes mentioned in the methods were reported in the results
Other bias	Unclear risk	Sources of funding: Not mentioned if they had any role in design or results of study

Bobat 2005

Methods	Randomised, double-blind, placebo-controlled trial conducted in Grey's Hospital in Pietermaritburg, South Africa. Baseline measurements of plasma HIV-1 viral load and the percentage of CD4T lymphocytes were established at two study visits before randomisation, and measurements were repeated 3, 6, and 9 months after the start of supplementation.
Participants	96 children with HIV-1 infection between the ages of 6 months and 60 months, being cared for as outpatients at Grey's Hospital, and not receiving anti-retroviral therapy were recruited. Pneumonia was diagnosed by history and physical examination, including chest auscultation, and confirmed by chest radiograph.
Interventions	Children either received 10 mg of elemental zinc as sulphate or placebo every day for 6 months. The child's parent or guardian was given one packet at the first two visits and two packets at each monthly follow-up visit thereafter, and was instructed on how to give the tablet.
Outcomes	The primary outcome measure was plasma HIV-1 viral load and incidence of pneumonia.
Notes	Outpatient management of children with HIV-1 infection is provided by a team of paediatricians, medical officers, and nurses who care for about 20 to 30 children per week. After starting zinc or placebo, children were assessed at Grey's Hospital every 2 weeks for the first month, monthly for 5 months, and a final visit 9 months after zinc or placebo supplementation started. Pneumonia was diagnosed by history and physical examination, including chest auscultation, and confirmed by chest radiograph. Funding: This study was funded by the Johns Hopkins Family Health and Child Survival Cooperative Agreement with the Office of Health, Infectious Diseases, and Nutrition, Global Health Bureau, US Agency for International Development.

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Quote: "Children were block–randomised in three age strata (6 to 23, 24 to 41, and 42 to 60 months)"; "Randomisation lists were computer generated at the WHO in a fixed block size of eight"
Allocation concealment (selection bias)	Unclear risk	Quote: "Tablets of zinc sulphate or placebo were produced by the same manufacturer (Nutriset, Bierne, France) and supplied in blister packets of 14 dispersible tablets"; "An investigator at Grey's Hospital assigned children to the treatment groups. The investigators were unaware of the treatment allocation until follow up was completed"

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Bobat 2005 *(Continued)*

Blinding of participants and personnel (performance bias) All outcomes	Low risk	Quote: "An investigator at Grey's Hospital assigned children to the treatment groups. The investigators were unaware of the treatment allocation until follow up was completed."
Blinding of outcome assessment (detection bias) All outcomes	Unclear risk	Insufficient information to permit judgement
Incomplete outcome data (attrition bias) All outcomes	Low risk	9/105 participants (8.6%) were excluded. 2/46 (4.4%) and 9/50 (18%) participants did not complete the trial in zinc and placebo groups, respectively. The reasons for lost to follow-up were mainly deaths and refused to participate
Selective reporting (reporting bias)	Low risk	We could not locate the protocol of this study. We could not find the trial registration number of the study. The outcomes mentioned in the methods were reported in the results
Other bias	Low risk	Sources of funding: funding agencies had no role in views and opinions mentioned in the study

Brooks 2005

Methods	RCT in which random assignment to zinc or placebo was done with permuted blocks of variable length between 2 and 8. Placebo was designed to be identical to the zinc syrup in colour, odour, and taste. The study was conducted at Kamalapur, southeastern Dhaka, Bangladesh. The medical officer diagnosed pneumonia if crepitations were heard on inspiration with a respiratory rate greater than 50 breaths per minute; severe pneumonia was diagnosed if there was also chest indrawing, or at least one other danger sign.
Participants	Children aged 60 days to 12 months at the time of enrolment and excluded those with known or suspected tuberculosis, chronic respiratory or congenital heart disease, or severe malnutrition requiring hospital admission. Pneumonia was diagnosed if crepitations were heard on inspiration with a respiratory rate greater than 50 breaths per minute; severe pneumonia was diagnosed if there was also chest indrawing, or at least one other danger sign. Children with wheezing or rhonchi with crepitations were also diagnosed with pneumonia. 809 children were randomly assigned to zinc and 812 to placebo. There were no significant differences between groups at baseline, except for a slightly higher proportion of boys in the zinc group. There was no difference between the groups in serum zinc values at baseline.
Interventions	Zinc was given orally as a syrup (35 mg zinc acetate per 5 mL). The placebo was non-nutritious and vitamin-free. Compliance required intake of two teaspoons of syrup (10 mL). Children with pneumonia were treated with co-trimoxazole (10 mg/kg trimethoprim, twice daily for 5 days) for pneumonia. Children on antibiotics were assessed within 72 hours of starting treatment; those who did not improve (i.e. the respiratory rate did not change by more than 5 breaths/minute from baseline) were switched to treatment with amoxicillin (40 mg/kg, three times daily for 5 days). If oral treatment failed, or if they had severe pneumonia, children were referred to hospital for parenteral treatment (ceftriaxone 75 mg/kg intramuscularly per day). Children with only expiratory wheezes or rhonchi were managed with salbutamol syrup (0.3 mg/kg, 3 times daily), or referred to hospital for danger signs.
Outcomes	Pneumonia incidence. Other outcomes included frequency of other illnesses and mortality.
Notes	Sources of funding: The research was funded by Johns Hopkins Family Health and Child Survival Cooperative Agreement with the US Agency for International Development, the Swiss Development Corporation, and a cooperative agreement between the US Agency for International Development (HRN-A-00-96-90005-00) and core donors to the Centre for Health and Population Research.

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Brooks 2005 *(Continued)*

Risk of bias

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "Random assignment to zinc or placebo was done with permuted blocks of variable length between two and eight"
Allocation concealment (selection bias)	Unclear risk	Insufficient information to permit judgement
Blinding of participants and personnel (performance bias) All outcomes	Low risk	Quote: "ACME Laboratories (Dhaka) prepared, labelled and masked the identity of both preparations. Both placebo and treatment were designed to be identical in colour, odour, and taste"; "identity of both the preparations were masked"
Blinding of outcome assessment (detection bias) All outcomes	Low risk	Quote: "Blinding of FRAs was not affected because a proportion of children in both zinc and placebo groups reacted to the taste such that the treatment could not be distinguished"
Incomplete outcome data (attrition bias) All outcomes	Low risk	Loss to follow-up was 9.1%. Withdrawal from both groups was most commonly attributed to the child's reaction to the taste of the syrup, which sometimes resulted in regurgitation. Most of those who withdrew were young, primarily breast fed infants. The highest proportion (37·1%) of withdrawals for both groups occurred at age 2 months, with 77·1% younger than 6 months
Selective reporting (reporting bias)	Low risk	We could not locate the protocol of this study. We could not find the trial registration number of the study. The outcomes mentioned in the methods were reported in the results
Other bias	Low risk	Study seems to be free from other biases; the funding sources had no role in the study design, data collection, data analysis, interpretation of results, or decision to publish this research Funding: The research was funded by Johns Hopkins Family Health and Child Survival Cooperative Agreement with the US Agency for International Development, the Swiss Development Corporation, and a cooperative agreement between the US Agency for International Development (HRN-A-00-96-90005-00) and core donors to the Centre for Health and Population Research

Luabeya 2007

Methods	What was the study design? The study was conducted in northern KwaZulu-Natal Province, South Africa. Children were enrolled into the study by nurses at five government primary health care clinics. Pneumonia by maternal report was considered to have occurred if there was a history of either fast breathing or chest in-drawing. Confirmed pneumonia was defined as an elevated respiratory rate at rest measured by the fieldworker using WHO/UNICEF Integrated Management of Childhood Illness guidelines.
Participants	Add number of participants. Children eligible for study were 4 to 6 months old. Children were excluded from the study if they were: less than 60% of median weight-for-age using United States National Center for Health Statistics standards; had nutritional oedema; had received vitamin or micronutrient supplements in the previous month; had diarrhoea for more than seven days at the time of study enrolment; or were enrolled in another study of a clinical intervention. Confirmed pneumonia was defined as an elevated respiratory rate at rest measured by the fieldworker using WHO/UNICEF Integrated Management of Childhood Illness guidelines.

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Luabeya 2007 *(Continued)*

Interventions	The 3 treatment arms were: vitamin A alone; vitamin A plus zinc; and vitamin A, zinc and multiple micronutrients. All supplements were given daily at home from entry into the study until 24 months of age.
Outcomes	Diarrhoea, pneumonia Incidence? prevalence?
Notes	Funding: Supported by grants from the US National Institute of Health (1 UO1 AI45508-01, 1 K24 AI/HDO1671-01, D43TW05572-01 to Dr Bennish) and the Wellcome Trust (Wellcome 62925 to Dr Bennish and Wellcome 063009 to Dr Van den Broeck. The sponsor for the study was the host institution, the Africa Centre for Health and Population Studies, which gave discretion in the investigative team in study design, data analysis, manuscript preparation, and decisions on manuscript submission and publication.

Risk of bias

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "An allocation list was prepared using computer-generated random numbers and a block size of six"; assignment to the three treatment arms was done separately for three cohorts of children stratified by HIV status of child and mother: HIV-infected children and mothers; HIV-uninfected children of HIV-infected mothers; and HIV-uninfected children of HIV-uninfected mothers
Allocation concealment (selection bias)	Low risk	Quote: "The manufacturer prepared numbered packs of tablets corresponding to the allocation list. Children enrolled in the study were assigned by a study physician to one of the three study cohorts after results of the HIV tests became available. The physician then allocated the next pack of tablets from the blocks assigned to that cohort to the participant"
Blinding of participants and personnel (performance bias) All outcomes	Low risk	Quote: "Investigators, study staff and participants were blind to the treatment assignments"
Blinding of outcome assessment (detection bias) All outcomes	Low risk	Quote: "Investigators, study staff and participants were blind to the treatment assignments"
Incomplete outcome data (attrition bias) All outcomes	Low risk	Exclusion (12.7%) and attrition (8.1% in vitamin A + zinc and 8.9% in vitamin A group) data were reported along with their reasons. Thirty-seven children withdrew and one died before any home visits took place
Selective reporting (reporting bias)	Low risk	The study appears to be free of selective reporting. Trial Registration ClinicalTrials.gov NCT00156832. The outcomes mentioned in the methods were reported in the results
Other bias	Low risk	Sources of funding: The sponsor for the study was the host institution, the Africa Centre for Health and Population Studies, which gave discretion in the investigative team in study design, data analysis, manuscript preparation, and decisions on manuscript submission and publication

Penny 2004

Methods	This randomised, double-blind, placebo-controlled, community-based trial was carried out in Canto Grande, a shanty town on the outskirts of Lima, Peru. The study was carried out in two phases. During the first phase researchers evaluated the effect of zinc or multiple micronutrient supplementation

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Penny 2004 *(Continued)*

	on the recovery from persistent diarrhoea. During the second phase researchers assessed the effect of continued supplementation on morbidity from new infections during the following 6 months.
Participants	412 children aged 6 to 36 months with diarrhoea for 14 days were randomly assigned, after being stratified for breastfeeding status, to receive two weeks of daily supplementation with one of three indistinguishable supplements: placebo; 20 mg zinc daily as zinc gluconate (zinc group); or 20 mg zinc daily as zinc gluconate plus a mixture of other micronutrients, i.e. vitamins and minerals (zinc VM group). A subset of children consisting of the first 246 children enrolled who intended to remain in the study area subsequently received the same assigned supplement at one-half the initial daily dose (10 mg zinc daily) and continued under observation for a total of 6 months.
Interventions	Supplements were supplied as individual doses of a dry micronutrient mixture with added sugar, colouring and flavouring agents, which were dissolved in clean water in participants' homes and provided as a liquid beverage under the supervision of study personnel on Monday through Friday and by parents or other caregivers during the weekends. There were two intervention arms, zinc plus vitamins and minerals who were given 10 mg of zinc supplementation along with different combinations of mineral and vitamins. Another interventional arm was given zinc 10 mg and the control group was not given any supplementation.
Outcomes	Changes in plasma zinc, haematocrit, haemoglobin, plasma ferritin, pneumonia incidence.
Notes	In this review, groups with zinc and placebo are included for analysis. Examination included assessment of hydration status, measurement of rectal temperature and monitoring of respiratory rate, which was counted for 1 minute and repeated if the rate was greater than age-specific upper limits (50/minute for children aged 6 to 11 months and 40/minute for children aged 11 months). Children were referred to the study physician for diagnosis and treatment when the fieldworker or caregiver was concerned about the child's health status or if the child had any one of several predefined signs of illness, including fever, presentation or worsening of cough with elevated respiratory rate (i.e. fieldworker-defined acute lower respiratory infection), persistent diarrhoea, diarrhoea with signs of dehydration, or vomiting or skin conditions requiring diagnosis. Sources of funding: Supported primarily by the Thrasher Research Fund and the World Health Organization; additional funds were provided by the University of California Pacific Rim Program.

Risk of bias

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Unclear risk	Insufficient information to permit judgement
Allocation concealment (selection bias)	Unclear risk	Insufficient information to permit judgement
Blinding of participants and personnel (performance bias) All outcomes	Unclear risk	Quote: "double-masked"
Blinding of outcome assessment (detection bias) All outcomes	Unclear risk	Quote: "double-masked"
Incomplete outcome data (attrition bias) All outcomes	High risk	14/81 (17.3%) in zinc and 13/83 (15.7%) placebo groups lost to follow-up but no reasons were reported

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Penny 2004 *(Continued)*

Selective reporting (reporting bias)	Low risk	We could not locate the protocol of this study. We could not find the trial registration number of the study. The outcomes mentioned in the methods were reported in the results
Other bias	Unclear risk	Sources of funding: funding agencies had no role in views and opinions mentioned in the study

Sazawal 1998

Methods	Double-blind RCT in which the loss of follow-up was less than 2% The study was conducted in a low socioeconomic population of urban India.
Participants	Children, 6 to 35 months of age, presenting to a community-based clinic for acute diarrhoea and before enrolment, a parent of the child was given an explanation of the study and written informed consent was obtained. The baseline characteristics for the child-periods included in the analysis were similar between the two groups. The zinc group had 298 participants and the placebo one had 311.
Interventions	Children were randomised to receive either zinc or placebo in a liquid preparation containing vitamins A (800 units), B1 (0.6 mg), B2 (0.5 mg), B6 (0.5 mg), D3 (100 IU), and E (3 mg) and niacinamide (10 mg); the zinc preparation contained zinc gluconate (10 mg elemental zinc). The liquid preparation 5 mL was given daily for 6 months to all enrolled children; during diarrhoeal illness this was increased to 10 mL to provide for excess zinc losses.
Outcomes	Incidence and prevalence of ALRI. ALRI was diagnosed as using WHO criteria for respiratory disease episodes based on fast breathing alone. ALRI was also defined as child having cough and at least one assessment documenting: a) an elevated respiratory rate more than the age-specific value on both 1-minute estimations; and b) a recorded temperature of more than 101°F or lower chest indrawing.
Notes	Funding: This work was supported by grants from the WHO Diarrheal Disease Control Program, the Thrasher Research Fund, the Johns Hopkins Family Health and Child Survival Cooperative Agreement with funding from the US Agency for International Development and the US National Institutes of Health (R29 HD34724). The assistance of Ms Usha Dhingra and Mr Dharminder Kashyap in data management and of Sandoz India Ltd for providing the supplements is appreciated.

Risk of bias

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	Quote: "Randomisation schedules with permuted blocks of 10 were used for children"
Allocation concealment (selection bias)	Low risk	Quote: "Supplements were prepared and coded by Sandoz India Ltd (Mumbai). Both formulation were liquid preparations, similar in colour and taste"
Blinding of participants and personnel (performance bias) All outcomes	Low risk	Quote: "double-blind"; "The Code, which was kept by WHO personnel, was not available to the investigator until the end of the study; "both formulation were liquid preparations, similar in colour and taste"
Blinding of outcome assessment (detection bias) All outcomes	Unclear risk	Insufficient information to permit judgement

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Sazawal 1998 *(Continued)*

Incomplete outcome data (attrition bias) All outcomes	High risk	Exclusion and attrition rates with their reasons were not described in the study
Selective reporting (reporting bias)	Low risk	We could not locate the protocol of this study. We could not find the trial registration number of the study. The outcomes mentioned in the methods were reported in the results
Other bias	Unclear risk	Sandoz India provided the supplements. Not clear of their role and other funding agencies

ALRI: acute lower respiratory infection
IU: international unit
RCT: randomised controlled trial
WHO: World Health Organization

Characteristics of excluded studies *[ordered by study ID]*

Study	Reason for exclusion
Adhikari 2016	Wrong participants: included children with recurrent respiratory infections
Baqui 2002	Wrong duration: zinc supplementation was given for 2 weeks
Baqui 2003	Wrong diagnosis: ALRI was diagnosed if the child had reported symptoms of cough or difficulty in breathing with rapid breathing with or without chest indrawing
Bates 1993	Wrong mode of supplementation: zinc supplement was delivered in a fortified drink
Castillo-Duran 1987	Wrong duration; wrong outcomes: zinc was supplemented for 60 days; did not study effects on diarrhoea or respiratory illnesses
Chandyo 2010	Wrong duration: duration of intervention was 2 weeks
Feiken 2014	Wrong duration: duration of intervention was 10 days
Larson 2010	Wrong diagnosis: did not use this review's specific ARI definition
Lind 2004	Wrong diagnosis: considered ‘cough and fever’ as ALRI outcome
Lira 1998	Wrong intervention and outcomes: infants were recruited and supplemented from birth; short course supplementation was provided; only cough was reported
Long 2006	Wrong outcome: respiratory tract infection outcomes were defined as the occurrence of cough alone, cough and fever, or cough and rapid respiratory rate as reported by the mother
Malik 2014	Wrong diagnosis: considered "cough or cold with or without fever. ALRI was diagnosed if the child had symptoms of cough with difficult and/or rapid breathing or chest indrawing as informed by the caregiver" as ALRI
Mazoomar 2010	Wrong diagnosis: considered caregiver’s report of cough or difficulty in breathing along with rapid breathing
McDonald 2015	Wrong population: infants aged less than 2 months at start of intervention (5 to 7 weeks)

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Study	Reason for exclusion
Ninh 1996	Wrong diagnosis: respiratory outcome was cough and fever
Osendarp 2002	Wrong population: Infants were recruited and supplemented from 4 weeks of age
Rahman 2001	Wrong duration: supplementation was given for 2 weeks only
Reul 1997	Wrong diagnosis: respiratory infections were defined as the presence of at least two of the following symptoms: runny nose, cough, wheezing, difficulty breathing, or fever
Richard 2006	Wrong diagnosis: ALRI was reported by parent as presence of cough and rapid respiration
Rosado 1997	Wrong diagnosis: respiratory illness was presence of runny nose, common cold, sore throat or cough
Roy 1999	Wrong duration: zinc supplementation period was 2 weeks
Sampaio 2013	Wrong diagnosis: study used Brazilian Ministry of Health Criteria as ARI definition
Sanchez 2014	Wrong diagnosis: presence of two or more of the following symptoms as ARI: "Cough, runny nose, shortness of breath and sore throat two or more days duration"
Sempértegui 1996	Wrong duration: zinc supplementation period was 60 days
Soofi 2013	Wrong diagnosis: definition of ARI: " Signs (fast breathing, chest indrawing) of acute respiratory illness were recorded as reported by the mother. An acute respiratory illness episode was defined as a minimum of 2 days with signs followed by a significant interval of at least 7 days"
Sur 2003	Wrong population: Infants were recruited and supplemented from within 7 days of birth
Taneja 2009	Wrong population: zinc supplementation was given to infants between 2 to 4 weeks and 12 months of age
Tielsch 2007	Wrong diagnosis and population: trial was on children aged 1 to 35 months with data not stratified by age, ARTI: episodes of acute respiratory illness were defined as one or more consecutive days of fever, cough, or difficulty breathing, with all three symptoms on at least 1 day during the episode and at least 7 days between episodes
Umeta 2000	Wrong outcomes: cough was only reported respiratory outcome
Vakili 2009	Wrong population: children included older than review's specified cut off (78 to 120 months)

ALRI: acute lower respiratory infection
ARI: acute respiratory infection
ARTI: acute respiratory tract infection

DATA AND ANALYSES

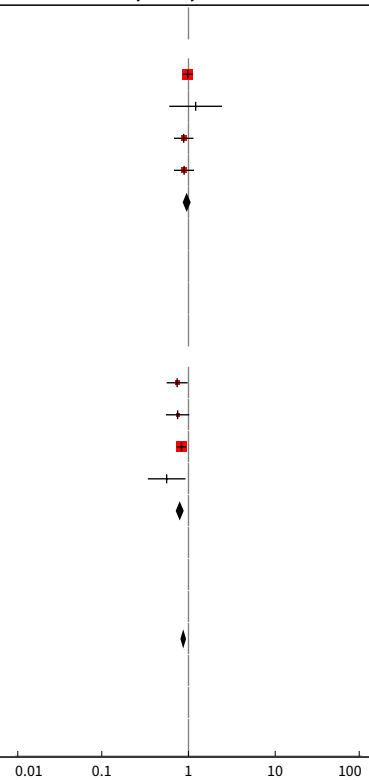
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Comparison 1. Zinc supplementation vs placebo

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1 Pneumonia incidence	6	5193	Risk Ratio (Fixed, 95% CI)	0.87 [0.81, 0.94]
1.1 Diagnosis based on age-specific fast breathing with or without lower chest indrawing	4	1932	Risk Ratio (Fixed, 95% CI)	0.95 [0.86, 1.06]
1.2 Diagnosis based age-specific fast breathing and confirmed by chest examination or chest radiograph	4	3261	Risk Ratio (Fixed, 95% CI)	0.79 [0.71, 0.88]
2 Pneumonia prevalence	1	609	Risk Ratio (Fixed, 95% CI)	0.59 [0.35, 0.99]

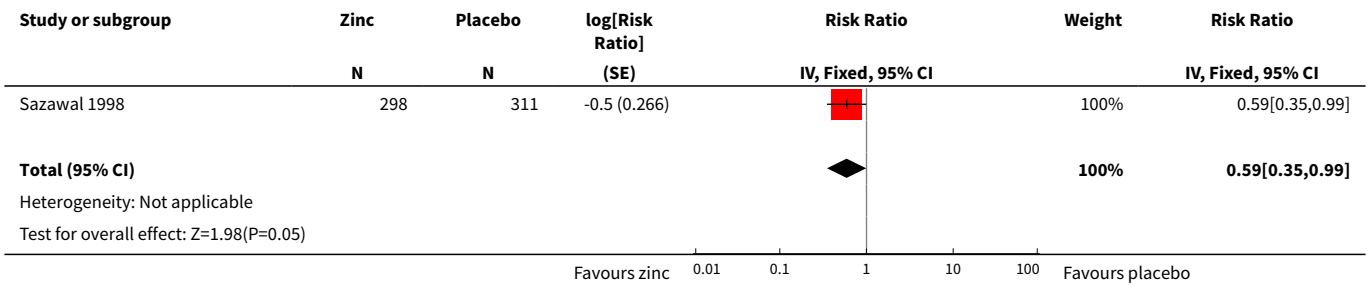
Analysis 1.1. Comparison 1 Zinc supplementation vs placebo, Outcome 1 Pneumonia incidence.

Study or subgroup	Favours experimental N	Placebo N	log[Risk Ratio] (SE)	Risk Ratio IV, Fixed, 95% CI	Weight	Risk Ratio IV, Fixed, 95% CI
1.1.1 Diagnosis based on age-specific fast breathing with or without lower chest indrawing						
Bhandari 2002	621	621	-0 (0.067)		32.75%	0.98[0.86,1.12]
Luabeya 2007	113	113	0.2 (0.358)		1.15%	1.21[0.6,2.44]
Penny 2004	80	79	-0.1 (0.131)		8.57%	0.88[0.68,1.14]
Sazawal 1998	149	156	-0.1 (0.137)		7.83%	0.89[0.68,1.16]
Subtotal (95% CI)					**50.29%**	**0.95[0.86,1.06]**
Heterogeneity: Tau^2=0; Chi^2=1.24, df=3(P=0.74); I^2=0%						
Test for overall effect: Z=0.9(P=0.37)						
1.1.2 Diagnosis based age-specific fast breathing and confirmed by chest examination or chest radiograph						
Bhandari 2002	620	620	-0.3 (0.142)		7.29%	0.74[0.56,0.98]
Bobat 2005	46	50	-0.3 (0.158)		5.89%	0.75[0.55,1.02]
Brooks 2005	809	812	-0.2 (0.066)		34.26%	0.83[0.73,0.94]
Sazawal 1998	149	155	-0.6 (0.255)		2.27%	0.56[0.34,0.92]
Subtotal (95% CI)					**49.71%**	**0.79[0.71,0.88]**
Heterogeneity: Tau^2=0; Chi^2=2.71, df=3(P=0.44); I^2=0%						
Test for overall effect: Z=4.29(P<0.0001)						
Total (95% CI)					**100%**	**0.87[0.81,0.94]**
Heterogeneity: Tau^2=0; Chi^2=9.74, df=7(P=0.2); I^2=28.09%						
Test for overall effect: Z=3.66(P=0)						
Test for subgroup differences: Chi^2=5.78, df=1 (P=0.02), I^2=82.69%						



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Analysis 1.2. Comparison 1 Zinc supplementation vs placebo, Outcome 2 Pneumonia prevalence.



ADDITIONAL TABLES

Table 1. Zinc supplement schedule and duration

Study	Supplement		Schedule	Duration	Surveillance
	Zinc	Control			
Bhandari 2002	**Zinc gluconate** 10 mg	Both groups vitamin A	Daily	4 months	Once weekly
Bobat 2005	**Zinc sulphate** 10 mg	Placebo	Daily	6 months	Every 2 weeks
Brooks 2005	**Zinc acetate** 35 mg to infants 70 mg to children aged > 12 months	Placebo	Weekly	12 months	Once weekly
Luabeya 2007	**Zinc gluconate** 10 mg	Both groups vitamin A	Daily	(Continued until 24 months of age)	Once weekly
Penny 2004	**Zinc gluconate** 10 mg	Placebo	Daily	6 months	Once weekly
Sazawal 1998	**Zinc gluconate** 10 mg	Placebo	Daily	4 months	Every 5th day

APPENDICES

Appendix 1. Previous search strategy

We searched the Cochrane Central Register of Controlled Trials (CENTRAL) (The Cochrane Library 2010, issue 2), which contains the Acute Respiratory Infections Group's Specialised Register, MEDLINE (1966 to January Week 2, 2010), Embase (1974 to January 2010) and LILACS (1985 to January 2010).

We used the following search strategy to search MEDLINE and CENTRAL. The search strategy incorporates the search strategy devised by Boluyt 2008 to identify child studies. Due to the small number of search results we chose not to use a search filter to identify randomised trials. We adapted the search strategy to search Embase (see Appendix 2) and LILACS (see Appendix 3).

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MEDLINE (Ovid)

1 exp Pneumonia/
2 pneumon*.tw.
3 lower respiratory tract infection*.tw.
4 lower respiratory infection*.tw.
5 lrti.tw.
6 or/1-5
7 Zinc/
8 (zinc or zn).tw,nm.
9 or/7-8
10 exp Infant/
11 (infant* or infancy or newborn* or baby* or babies or neonat* or preterm* or prematur*).tw.
12 exp Child/
13 (child* or schoolchild* or school age* or preschool* or kid or kids or toddler*).tw.
14 Adolescent/
15 (adoles* or teen* or boy* or girl*).tw.
16 Minors/
17 Puberty/
18 (minor* or pubert* or pubescen*).tw.
19 exp Pediatrics/
20 (pediatric* or pediatric*).tw.
21 exp Schools/
22 (nursery school* or kindergar* or primary school* or secondary school* or elementary school* or high school* or highschool*).tw.
23 or/10-22
24 6 and 9 and 23

Embase search strategy

14. #5 AND #9 AND #13
13. #10 OR #11 OR #12
12. child*:ab,ti OR schoolchild*:ab,ti OR preschool*:ab,ti OR kid:ab,ti OR kids:ab,ti OR toddler*:ab,ti OR pediatric*:ab,ti OR paediatric*:ab,ti OR kindergar*:ab,ti OR (school* NEAR/2 (nursery OR primary OR elementary OR age*)):ab,ti
11. infant*:ab,ti OR infancy:ab,ti OR newborn*:ab,ti OR baby*:ab,ti OR babies:ab,ti OR neonat*:ab,ti OR preterm*:ab,ti OR prematur*:ab,ti
10. 'infant'/exp OR 'child'/exp OR 'pediatrics'/exp OR 'school'/exp
9. #6 OR #7 OR #8
8. zinc:ab,ti OR zn:ab,ti
7. 'gluconate zinc'/exp
6. 'zinc'/exp
5. #1 OR #2 OR #3 OR #4
4. 'lower respiratory tract infection':ab,ti OR 'lower respiratory tract infections':ab,ti OR 'lower respiratory infection':ab,ti OR 'lower respiratory infections':ab,ti OR lrti:ab,ti
3. 'lower respiratory tract infection'/de
2. pneumon*:ab,ti
1. 'pneumonia'/exp

LILACS search strategy

pneumon$ or namonia or pulmonia or neumonia [Words] and zinc$ or cinc [Words]

"PNEUMONIA" [Subject descriptor] and "ZINC" [Subject descriptor]

Appendix 2. MEDLINE (Ovid) search strategy

1 exp Pneumonia/
2 (pneumon* or bronchopneumon* or pleuropneumon*).tw.
3 ((respiratory tract infect* or respiratory infect*) adj3 lower).tw.
4 (lower respiratory adj3 infect*).tw.
5 lrti.tw.
6 or/1-5
7 Zinc/
8 (zinc* or zn).tw,nm.
9 Dietary Supplements/

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10 trace elements/ or zinc/
11 Micronutrients/
12 exp Zinc Compounds/
13 ((essential or trace) adj1 (mineral* or nutrient*)).tw.
14 micronutrient*.tw.
15 or/7-14
16 6 and 15

Appendix 3. Embase.com search strategy

#24 #15 AND #23
#23 #18 NOT #22
#22 #19 NOT #21
#21 #19 AND #20
#20 'human'/de AND [embase]/lim
#19 'animal'/de OR 'nonhuman'/de OR 'animal experiment'/de
#18 #16 OR #17
#17 random*:ab,ti OR placebo*:ab,ti OR crossover*:ab,ti OR 'cross over':ab,ti OR allocat*:ab,ti OR trial:ti OR (doubl* NEXT/1 blind*):ab,ti
#16 'randomized controlled trial'/exp OR 'single blind procedure'/exp OR 'double blind procedure'/exp OR 'crossover procedure'/exp
#15 #6 AND #14
#14 #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13
#13 micronutrient*:ab,ti
#12 ((essential OR trace) NEAR/1 (mineral* OR nutrient*)):ab,ti
#11 'zinc derivative'/de
#10 'trace element'/exp
#9 'diet supplementation'/de
#8 zinc*:ab,ti OR zn:ab,ti
#7 'zinc'/de
#6 #1 OR #2 OR #3 OR #4 OR #5
#5 lrti:ab,ti
#4 'lower respiratory tract infection':ab,ti OR 'lower respiratory tract infections':ab,ti OR 'lower respiratory infection':ab,ti OR 'lower respiratory infections':ab,ti
#3 'lower respiratory tract infection'/de
#2 pneumon*:ab,ti OR bronchopneumon*:ab,ti OR pleuropneumon*:ab,ti
#1 'pneumonia'/exp

Appendix 4. LILACS (Virtual Health Library) search strategy

> Search > (MH:pneumonia OR pneumon$ OR Neumonía OR MH:C08.381.677$ OR MH:C08.730.610$ OR Pulmonía OR Bronchopneumonia OR Bronconeumonía OR Broncopneumonia OR Pleuropneumonia OR Pleuroneumonía OR MH:"Respiratory Tract Infections" OR "Infecciones del Sistema Respiratorio" OR "Infecções Respiratórias" OR "lower respiratory infection" OR "lower respiratory infections" OR "lower respiratory tract infection" OR "lower respiratory tract infections" OR lrti) AND (MH:zinc OR zinc OR zinco OR zn OR MH:"Zinc Compounds" OR MH:D01.975$ OR MH:"Dietary Supplements" OR "Suplementos Dietéticos" OR "Suplementos Dietéticos" OR MH:"Trace Elements" OR Oligoelementos OR MH:Micronutrients OR Micronutrientes)

Appendix 5. CINAHL (EBSCO) search strategy

S23 S13 and S22
S22 S14 or S15 or S16 or S17 or S18 or S19 or S20 or S21
S21 (MH "Quantitative Studies")
S20 TI placebo* OR AB placebo*
S19 (MH "Placebos")
S18 TI random* OR AB random*
S17 TI ((singl* or doubl* or tripl* or trebl*) N1 (blind* or mask*)) OR AB ((singl* or doubl* or tripl* or trebl*) N1 (blind* or mask*))
S16 TI clinic* trial* OR AB clinic* trial*
S15 PT clinical trial Search modes -
S14 (MH "Clinical Trials+")
S13 S4 and S12
S12 S5 or S6 or S7 or S8 or S9 or S10 or S11
S11 TI micronutrient* OR AB micronutrient*
S10 TI ((essential or trace) N1 (mineral* or nutrient*)) OR AB ((essential or trace) N1 (mineral* or nutrient*))
S9 (MH "Micronutrients")
S8 (MH "Trace Elements")

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S7 (MH "Dietary Supplements")
S6 TI (zinc* or zn) OR AB (zinc* or zn)
S5 (MH "Zinc") OR (MH "Zinc Compounds+")
S4 S1 or S2 or S3
S3 TI (lower respiratory tract infect* or lower respiratory infect* or lrti) OR AB (lower respiratory tract infect* or lower respiratory infect* or lrti)
S2 TI (pneumon* or bronchopneumon* or pleuropneumon*) OR AB (pneumon* or bronchopneumon* or pleuropneumon*)
S1 (MH "Pneumonia+")

Appendix 6. Web of Science (Thomson Reuters)

Topic=(pneumonia or bronchopneumonia or pleuropneumonia) AND Topic=(zinc)
Refined by: Topic=(random* or placebo* or clinical trial* or double blind*)
Timespan=All Years. Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC.
Lemmatization=Off

F E E D B A C K

Feedback comment by Joseph L. Mathew, 10 February 2011

Summary

Please note the following feedback.

1. The forest plot in Analysis 1.1 shows that participants in the trial by Bhandari et al, have been split to present two different outcomes. Half the participants in each arm are included for the outcome "Diagnosis based on age-specific fast breathing with or without lower chest indrawing" and half for the outcome "Diagnosis based age-specific fast breathing AND confirmed by chest examination or chest radiograph". Both outcomes have been presented as subgroup analysis in the same forest plot; although the trial report mentions that both outcomes were evaluated in all participants. Please confirm whether the procedure adopted in this review is standard practice; and the purpose of doing this.

Answer: We presented the findings after dividing the participants equally in the four cells so that participants do not repeat across analysis. The number of participants were 1241 in each group and we divided them as 620/621 for each of the four cells).

2. A similar adjustment seems to have been made with the participants in the trial by Sazawal et al.

Answer: Adjusted

3. The reference section mentions three citations under Sazawal 1998a; and one under Sazawal 1998b. The third citation in Sazawal 1998a is the same as Sazawal 1998b. Further, none of the citations reports 3300 participants in the trial; whereas this is the number included in the meta-analysis. Sazawal's 1995 publication in the New England Journal of Medicine mentions 937 participants; while the 1998 publication in Pediatrics reported 609 participants. Please confirm the basis for using the numbers in the meta-analysis.

Answer: Those were the two parts of the same study. We have now merged it and presented it as one study.

4. The meta-analysis has pooled data from children with HIV infection, along with otherwise healthy children. While it seems reasonable to pool children with unknown HIV status with otherwise healthy children, it would be better to analyse HIV infected children separately since pneumonia in immuno-compromised children could be quite different from otherwise healthy children.

Answer: In the text only, we presented the results on removing Bobat trial as well.

Submitter agrees with default conflict of interest statement:
I certify that I have no affiliations with or involvement in any organization or entity with a financial interest in the subject matter of my feedback.

Reply

We thank Dr Mathew for these comments.

Contributors

Joseph L. Mathew

W H A T ' S N E W

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Date	Event	Description
25 January 2017	Amended	Minor data transcription error corrected

HISTORY

Protocol first published: Issue 2, 2006
Review first published: Issue 12, 2010

Date	Event	Description
26 October 2015	New search has been performed	A new review author joined us to update this review. We excluded 11 new trials (Adhikari 2016; Chandyo 2010; Feiken 2014; Larson 2010; Malik 2014; Mazoomar 2010; McDonald 2015; Sampaio 2013; Sanchez 2014; Soofi 2013; Vakili 2009).
26 October 2015	New citation required but conclusions have not changed	Our conclusions remain unchanged.
26 October 2015	New search has been performed	Searches updated,
8 May 2013	New search has been performed	Searches conducted
10 February 2012	Amended	Authors replied to Feedback comment
8 August 2011	Feedback has been incorporated	Feedback added to review.

CONTRIBUTIONS OF AUTHORS

Zohra S Lassi (ZSL) entered data, created the comparisons, carried out the analysis and wrote the text of the review under the guidance of Dr Zulfiqar A Bhutta (ZAB). Anoosh Moin (AM) took part in screening results and updating the review.

DECLARATIONS OF INTEREST

Zohra S Lassi: none known.

Anoosh Moin: none known.

Zulfiqar A Bhutta: none known.

SOURCES OF SUPPORT

Internal sources

- The Aga Khan University, Pakistan.

External sources

- No sources of support supplied

DIFFERENCES BETWEEN PROTOCOL AND REVIEW

We have added mandatory sections such as subgroup analysis and sensitivity analysis for this update.

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INDEX TERMS

Medical Subject Headings (MeSH)

Gluconates [administration & dosage]; Pneumonia [epidemiology] [*prevention & control]; Randomized Controlled Trials as Topic; Zinc Acetate [administration & dosage]; Zinc Compounds [*administration & dosage]; Zinc Sulfate [administration & dosage]

MeSH check words

Child, Preschool; Humans; Infant

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FOI 26-3132 - Document 5

AAC Journals.ASM.org

Anthelmintic Avermectins Kill *Mycobacterium tuberculosis*, Including Multidrug-Resistant Clinical Strains

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Department of Microbiology and Immunology, and Centre for Tuberculosis Research, Life Sciences Centre, University of British Columbia, Vancouver, British Columbia, Canada[a]; Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA[b]

Avermectins are a family of macrolides known for their anthelmintic activities and traditionally believed to be inactive against all bacteria. Here we report that members of the family, ivermectin, selamectin, and moxidectin, are bactericidal against mycobacterial species, including multidrug-resistant and extensively drug-resistant clinical strains of *Mycobacterium tuberculosis*. Avermectins are approved for clinical and veterinary uses and have documented pharmacokinetic and safety profiles. We suggest that avermectins could be repurposed for tuberculosis treatment.

Antibacterial drug discovery is a costly endeavor with a very limited probability of success (1). The need for new therapies is especially acute in the case of tuberculosis (TB). While *Mycobacterium tuberculosis* is notoriously resistant to most antibiotics and new drugs with novel modes of action are urgently needed, such compounds have proven to be rare and difficult to identify (2). An alternative approach to generate new TB treatment options in a timely and cost-effective manner is "repurposing," i.e., identifying new applications for existing, clinically approved drugs (alone or in combination) with known pharmaceutical properties (3). In a recent study, we validated the concept that drugs that do not inhibit *M. tuberculosis* at clinically relevant concentrations might be introduced for TB therapy if they could be administered within a synergistic combination (4).

In the course of this screening program, we found that selamectin, a commonly used anthelminthic veterinary drug of the avermectin family, effectively inhibited mycobacterial growth in agar and liquid cultures. The avermectins were discovered in the mid-1970s in an antinematode screening program led by Kitasato Institute and the Merck, Sharp, and Dohme (MSD) laboratories (5). The antimycobacterial activity we discovered was surprising because the avermectins were thought to be only effective against helminths, insects, and arachnids and to be inactive against flatworms, protozoa, bacteria, and fungi (5–9). However, we could not identify literature with specific information describing the (lack of) antibacterial activity of the avermectins. In all probability, these negative data remained proprietary information and were never published. We therefore examined the antibacterial effectiveness of four avermectins (doramectin, ivermectin, moxidectin, and selamectin) (Fig. 1) against representative Gram-positive and Gram-negative bacteria (Table 1). Inhibitory effects were not observed on any of these bacteria at concentrations as high as 256 μg/ml using the bacterial growth indicator MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. The avermectins were then tested for their inhibitory activities against various *Mycobacterium* species using the same MTT assay (Table 1). This assay has been previously used to test drug sensitivity of *M. tuberculosis*, and the results are entirely consistent with other well-established methods based on nitrate reductase (12), resazurin (12), [^{3}H]glycerol uptake (13), or the gold standard proportion method of viable CFU after treatment (12, 14). All four avermectins inhibited growth of *Mycobacterium bovis* BCG and *M. tuberculosis* laboratory strains (H37Rv, CDC 1551, and Erdman) at concentrations ranging from 1 to 8 μg/ml. Three of the four also inhibited growth of *Mycobacterium smegmatis* within this concentration range. All four avermectins were less active against *Mycobacterium avium*; doramectin had lower levels of activity against all *Mycobacterium* species. Recent reports have shown that the antimycobacterial activity of some drugs is dependent on the presence of glycerol in the assay medium, leading to the identification of leads that lack activity *in vivo* (15, 16). When the activities of ivermectin, moxidectin, and selamectin were assayed in the absence of glycerol against *M. tuberculosis*, only a slight decrease (twofold) in their MICs was observed, demonstrating a glycerol-independent mode of action for the avermectins. In summary, the avermectins were found to be active against each of the four *Mycobacterium* species tested, while all were inactive against bacteria belonging to diverse related and unrelated taxa. Although these structurally related compounds had detectable differences in their activities against the four *Mycobacterium* species, our results suggest that mycobacteria share an unknown essential target for avermectins.

In order to investigate the potential inclusion of avermectins in the limited repertoire of TB drugs that might be used against drug-resistant strains, the activities of avermectins were also surveyed against multidrug-resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* clinical isolates from different geographical locations (Table 1). The MICs of ivermectin, selamectin, and moxidectin were similar against a panel of 27 MDR and XDR clinical isolates having elevated drug resistance profiles including first- and second-line anti-TB drugs, such as ethambutol, ethionamide, isoniazid, kanamycin, rifabutin, rifampin, *p*-amino salicylate (PAS), pyrazinamide, and streptomycin (Table 1). Only three multidrug-resistant (CI15072, CI12081, and BC-MDR2) and two

Received 15 August 2012 **Returned for modification** 7 September 2012
Accepted 13 November 2012
Published ahead of print 19 November 2012
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S.R.-G. and C.J.T. contributed equally to this article.
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doi:10.1128/AAC.01696-12

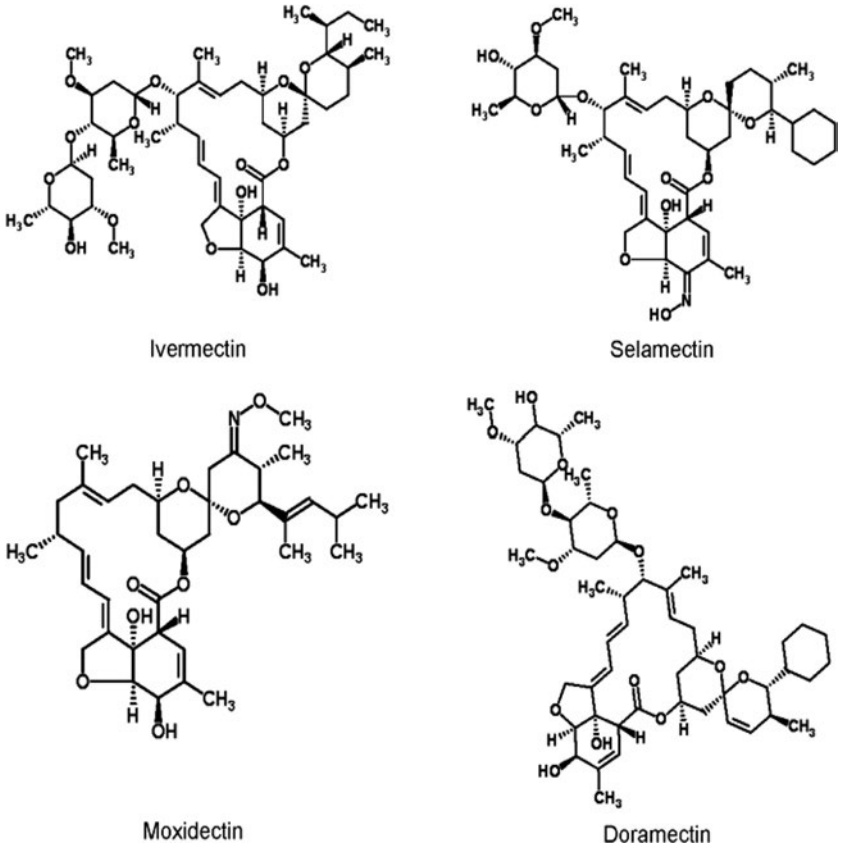


FIG 1 Avermectins used in this study. Images were obtained from ChemSpider.

drug-susceptible (BC-DS4 and BC-DS5) strains were less sensitive to ivermectin ($MIC_{90} > 24$ μg/ml). Nevertheless, inhibitory activity against these strains was reflected in low MIC_{50} values (<8 μg/ml). Importantly, the sensitivity of these strains to selamectin and moxidectin was unaffected. In summary, the avermectins were as effective against most drug-resistant *M. tuberculosis* clinical isolates as they were against *M. tuberculosis* laboratory strains.

To address the question of whether avermectins are bactericidal or bacteriostatic, survival kinetic experiments were done for ivermectin, selamectin, and moxidectin (Fig. 2). Two experiments performed independently, under similar but not identical growth conditions (see the Fig. 2 legend), measured kill kinetics. In the first experiment (Fig. 2A), 21-day kill curves were performed using various concentrations of ivermectin, selamectin, and moxidectin against the laboratory *M. tuberculosis* strain H37Rv. Here, selamectin showed the strongest bactericidal profile. All avermectins proved to be bactericidal, reducing initial bacterial viability up to 6 orders of magnitude (the limits of CFU detection). In the comparable kill kinetic experiment (Fig. 2B) performed independently at another location, the activity of the same dose (20 μg/ml) of each of the avermectins was measured over the same time period against *M. tuberculosis* H37Rv and $mc^2$5857 (an MDR strain; see Table 1 for details). Results were consistent with those described above (Fig. 2A). Moreover, all avermectins tested showed promising bactericidal activity against the MDR strain, reinforcing the MIC data and suggesting potential application of the avermectins for the treatment of MDR and XDR TB patients.

The *in vitro* pharmacodynamic parameters of the avermectins were further analyzed in a third experiment (Fig. 3). *In vitro* dose-response curves for the avermectins were obtained by plotting the change in $\log_{10}$ CFU of the inoculum against the drug concentration *C*/MIC. Using an alternative method to visualize kill kinetics, each avermectin concentration was multiplied by the time of exposure ($C \times T_{days}$) and then divided by the MIC to give the *in vitro* area under the concentration-time curve (AUC/MIC ratio), a standard measure of drug exposure. These analyses both showed that avermectins had exposure-dependent kill kinetics against *M. tuberculosis* under standard *in vitro* broth conditions. The AUC/MIC needed to achieve 1 $\log_{10}$ CFU/ml reduction varied between 2 and 4, while a bactericidal effect (4 $\log_{10}$ CFU/ml reduction, 99.99% killing) required AUC/MIC ratios between 12 and 14. From a TB drug treatment perspective, this value indicates the strong bactericidal effect of avermectins relative to rifampin, the standard frontline drug. The AUC at 24 h (AUC_{24})/MIC value required for a comparable 1 $\log_{10}$ CFU/ml reduction of the first-line antituberculosis drug rifampin is more than 10 times higher (18).

Ivermectin has been used to treat human onchocerciasis and lymphatic filariasis for over 20 years (19–21). Other avermectins (doramectin, moxidectin, and selamectin) used in veterinary medicine for nematode control in pets and livestock are known to be safe and well-tolerated for these indications (19). Selamectin can be administered topically, subcutaneously, or orally in the

FOI 26-3132 - Document 5

TABLE 1 Antimicrobial activities of four avermectins against bacterial species, including multidrug-resistant *M. tuberculosis* clinical isolates[a]

Species	Strain[b]	Drug resistance profile[c]	*In vitro* MIC_{90} (μg/ml) for:			
			Ivermectin	Selamectin	Moxidectin	Doramectin[d]
Escherichia coli	O157:H7	WT	>256	>256	>256	>256
Acinetobacter baumannii	ATCC 1960	WT	>256	>256	>256	>256
Pseudomonas aeruginosa	PA01 (H103)	WT	>256	>256	>256	>256
S. lividans	1326	WT	>256	>256	>256	>256
R. jostii	RHA1	WT	>256	>256	>256	>256
Kocuria rhizophila		WT	>256	>256	>256	>256
Staphylococcus aureus	ATCC 25923	WT	>256	>256	>256	>256
M. smegmatis	mc^2155	WT	8	4	4	128
M. avium	ATCC 25291	WT	>128	16	>128	>128
M. bovis	BCG Pasteur	WT	4	4	4	8
M. tuberculosis	H37Rv	WT	6	3	3	8
M. tuberculosis	CDC 1551	WT	4–8	1	2	4–8
M. tuberculosis	Erdman	WT	8	2	2–4	8
M. tuberculosis	1254		8	2–4	4	8–16
M. tuberculosis	H37Rv mc^24977	INH	6	1.5	3	ND
M. tuberculosis	CI5447	INH	1.5	1.5	0.8–1.5	ND
M. tuberculosis	CI5297	INH	6	3	6	ND
M. tuberculosis	CI5305	INH	3	1.5	1.5	ND
M. tuberculosis	H37Rv mc^25857	INH, RIF	3	1.5	3	ND
M. tuberculosis	H37Rv mc^25858	INH, RIF	3	1.5	3	ND
M. tuberculosis	V2475	INH, RIF	1.5	1.5	3	ND
M. tuberculosis	CI5058	INH, RIF	6	6	6	ND
M. tuberculosis	CI5324	INH, RIF	6	3	3	ND
M. tuberculosis	CI5400	INH, RIF	3	3	3	ND
M. tuberculosis	CI5072	INH, RIF, SM	>24	3–6	3	ND
M. tuberculosis	CI5358	INH, EMB, ETH	3	1.5	3	ND
M. tuberculosis	CI5459	INH, EMB, ETH	3	1.5–3	1.5	ND
M. tuberculosis	CI12081	INH, RIF, SM, EMB, ETH	>24	3	3	ND
M. tuberculosis	KZN11	INH, RIF, SM, EMB	3	3	3	ND
M. tuberculosis	KZN5	INH, RIF, SM, EMB, ETH, KM, PZA	6	3	3	ND
M. tuberculosis	KZN6	INH, RIF, SM, EMB, ETH, KM, PZA	6	6	3	ND
M. tuberculosis	KZN12	INH, RIF, SM, EMB, ETH, KM, PZA	3	3	1.5	ND
M. tuberculosis	KZN14	INH, RIF, SM, EMB, ETH, KM, PZA	6–12	6	3–6	ND
M. tuberculosis	KZN15	INH, RIF, SM, EMB, ETH, KM, PZA	6	3	3	ND
M. tuberculosis	KZN16	INH, RIF, SM, EMB, ETH, KM, PZA	3	1.5	1.5	ND
M. tuberculosis	BC-DS1		4–16	2	4	ND
M. tuberculosis	BC-DS3		8–16	1–2	2	ND
M. tuberculosis	BC-DS4		64 (8)	1–2	2	ND
M. tuberculosis	BC-DS5		>128 (8)	2–4	2–4	ND
M. tuberculosis	BC-MDR2	INH, RIF, PZA, SM, RBT	>128 (8)	2	2–4	ND
M. tuberculosis	BC-MDR3	INH, RIF, RBT	1–2	0.5–1	1–2	ND
M. tuberculosis	BC-MDR4	INH, RIF, EMB, PZA, RBT	4–16	2	2–4	ND
M. tuberculosis	BC-MDR5	INH, RIF, PZA, SM, PAS, RBT	4–16	2	2–4	ND

[a] Mycobacterial strains were assayed in liquid 7H9 media containing 0.2% glycerol and 10% albumin-dextrose-saline. Nonmycobacterial strains were assayed in LB medium. The MTT assay was used to determine the concentration that inhibited growth by 90%. Values in parentheses indicate 50% growth inhibition (MIC_{50}). The *M. smegmatis* strain was incubated for 3 days, the *M. avium*, *M. bovis* BCG, and *M. tuberculosis* strains were incubated for 7 days, and the nonmycobacterial strains were incubated overnight before the addition of MTT.

[b] Strains V2475 and 1254 have been described previously (10, 11). CI strains are clinical isolates from Mexico. KZN strains are clinical isolates from KwaZulu-Natal, South Africa. BC strains are clinical isolates from British Columbia, Canada.

[c] WT, wild type (no resistance mutations); EMB, ethambutol; ETH, ethionamide; INH, isoniazid; KM, kanamycin; RBT, rifabutin; RIF; rifampin; PAS, *p*-amino salicylate; PZA, pyrazinamide; SM, streptomycin.

[d] ND, not determined.

veterinary setting to treat a number of ecto- and endoparasite conditions in dogs and cats, and it is not presently approved for human use (22). Moxidectin is also safe in humans (23), and it is currently undergoing a phase III clinical trial to compare its efficacy with ivermectin in subjects with *Onchocerca volvulus* infection (http://clinicaltrials.gov/ct2/show/NCT00790998). Because ivermectin is extremely well-tolerated, effective, orally active, and associated with long-term safety, Merck & Co. has donated it to patients with river blindness in needy areas throughout the world (24). Furthermore, the success of this longstanding give-away program indicates that industrial production processes are established and ivermectin could be distributed to large populations with high incidences of MDR TB, a distinct advantage for implementation of TB drug therapy programs. However, to introduce

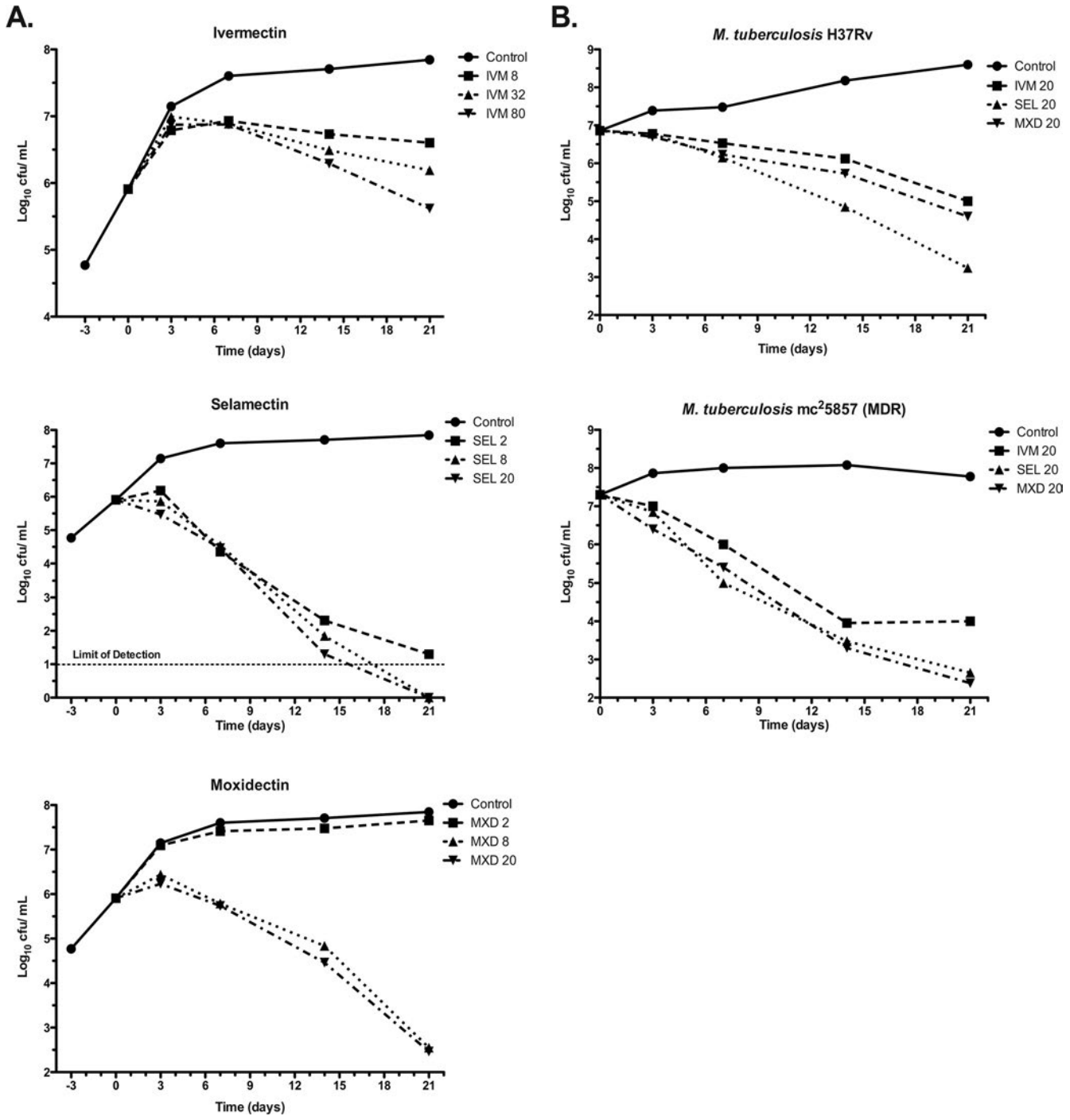


FIG 2 Time-kill kinetics of avermectins against *M. tuberculosis*. Two experiments were performed independently, at two different locations, under similar but not identical growth conditions. (A) Dose titration. Frozen stocks of *M. tuberculosis* H37Rv were cultured in 15 ml of 7H9 broth supplemented with 10% albumin-dextrose-catalase, 0.2% glycerol in standing 25-cm^2 tissue culture flasks at 37°C and 5% CO_2 for 3 days before the addition of avermectins. Cultures were agitated only during sampling. (B) Fixed dose strain comparison. Cultures (5 ml) of *M. tuberculosis* H37Rv and mc^25857 (an MDR isolate) were pregrown in shaking 35-ml bottles in 7H9 broth supplemented with 10% ADC, 0.2% glycerol, and 0.05% tyloxapol to an optical density at 600 nm (OD_{600}) of 0.8. To assess drug activity, cultures were washed once in phosphate-buffered saline (PBS), diluted (1/150) in the same medium (without tyloxapol), and grown at 37°C in shaking 15-ml conical tubes containing ivermectin, selamectin, or moxidectin at 20 μg/ml. Prior to plating, cell clumps were disrupted by sonication and viability was quantified by plating on 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase and 0.2% glycerol. Plates were incubated at 37°C, and colonies were counted after 2 weeks of incubation (microscopically) and reassessed after 4 weeks. Concentrations of avermectins are expressed in μg/ml. IVM, ivermectin; SEL, selamectin; and MXD, moxidectin.

FOI 26-3132 - Document 5

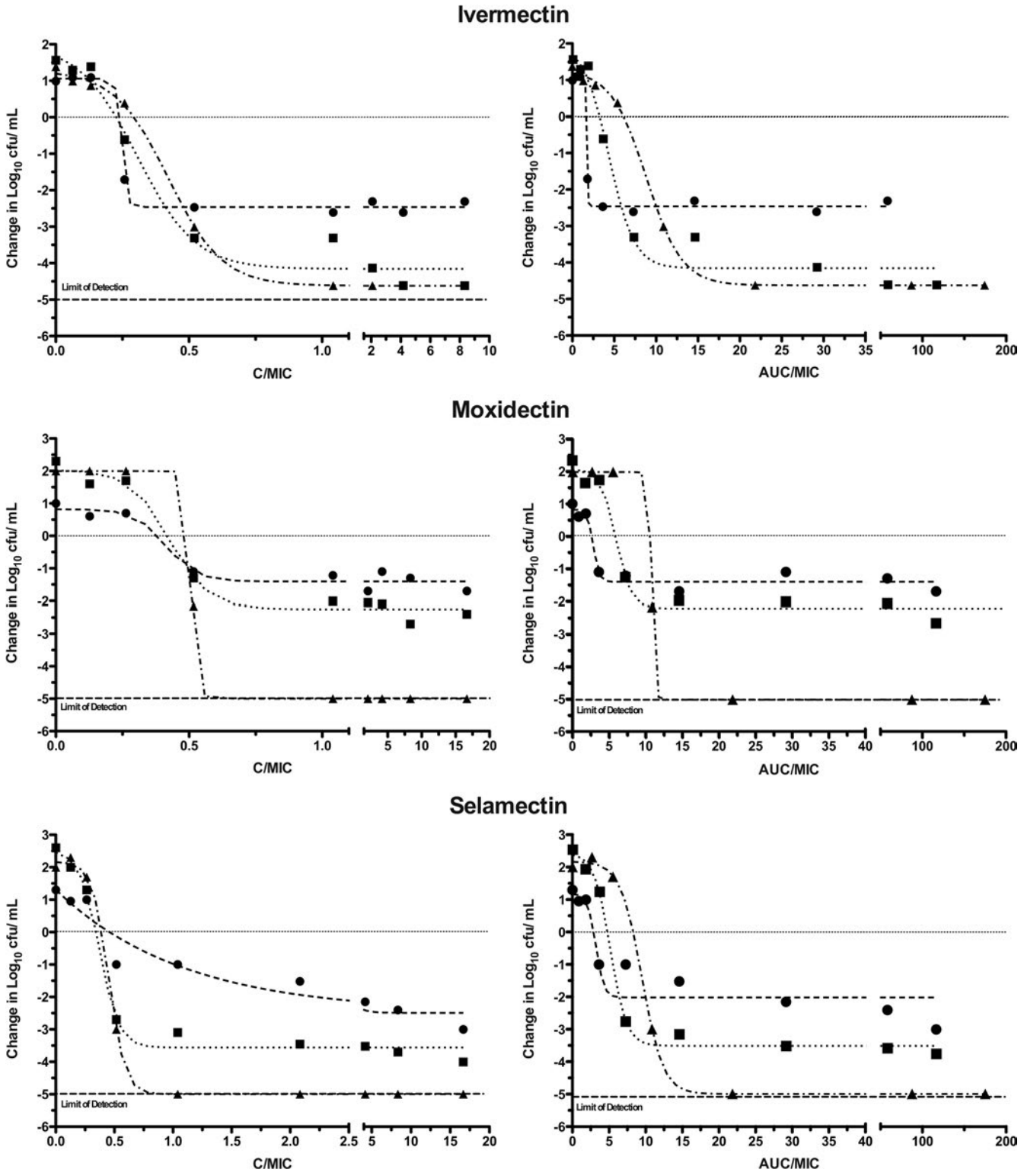


FIG 3 Effect of concentration and exposure on the bactericidal activities of avermectins against *M. tuberculosis*. Comparison of concentration and area under the curve over MIC ratios (C/MIC and AUC/MIC, respectively) on the bactericidal activities of the avermectins. The bactericidal effect was calculated on the basis of the initial inoculum prior to the addition of avermectins. Analysis of antimycobacterial activity was performed by comparing the rates of bacterial killing determined by nonlinear regression analysis with 95% confidence limits. The r^2 values on days 7 (filled circles), 14 (filled squares), and 21 (filled triangles) were 0.9959, 0.9766, and 0.9989 for ivermectin, 0.9591, 0.9827, and not available for moxidectin, and 0.88, 0.9949, and 0.9992 for selamectin, respectively. Cells were processed as described in the Fig. 2 legend for panel B but grown at 37°C in shaking 96-well plates containing 2-fold serial drug dilutions. The MIC values used for calculations were 6, 3, and 3 μg/ml for ivermectin, moxidectin, and selamectin, respectively, as calculated by the MTT assay.

any new TB therapy (especially for MDR or XDR TB), it is essential to establish that it is equal to or better than current treatment options; the promising 2- to 8-μg/ml range of MICs for avermectins in liquid cultures is comparable to that of second-line TB drugs, ranging from 1 to 25 μg/ml against *M. tuberculosis* H37Rv (25). The *in vitro* AUC/MIC ratios of avermectins are a pharmacodynamic predictor of effective bactericidal activity *in vivo*. Importantly, the low exposure needed to achieve this effect (AUC/MIC ratios of 10 to 15) indicates the potential of the avermectins for TB therapy. Thus, the avermectins might be readily adopted into the limited repertoire of drugs available to treat MDR and XDR TB. However, *in vivo* pharmacokinetic and efficacy studies will be needed to assess their clinical application for treating TB.

The apparent specificity of avermectins for mycobacteria implies that their target is unique to this taxon of bacteria. The remarkable complexity and uniqueness of the mycobacterial cell envelope, not present in other bacteria that are resistant to avermectins (including *Streptomyces lividans* and *Rhodococcus jostii*, both related actinobacteria), suggest this structure as a potential target (26). In invertebrate nematodes, avermectins specifically bind to glutamate-gated chloride channels present in nerve and muscle cells, causing paralysis and reduced ability to reproduce (27). Avermectins are orally active and distributed to most parts of the human body, including the lungs (24, 28). Ivermectin does not bind ligand-gated chloride channels in mammals, possibly due to impermeability of the blood-brain barrier and nonspecific pumps that prevent it from reaching the central nervous system (20, 27). Interestingly, moxidectin and selamectin, compounds used extensively in animals to treat a variety of parasitic indications without reported toxicity issues, were also highly active against *M. tuberculosis*. The specificity of avermectins for mycobacteria would be ideal for selectively targeting the pathogen while minimizing deleterious effects on resident gut flora after oral administration.

In summary, this is the first report demonstrating the antimycobacterial activity of avermectins. Their established safety profiles in humans and animals make them potential therapeutic options for treating TB. Future work will be done to identify the mycobacterial target of avermectins, to characterize pharmacokinetic and pharmacodynamic properties in animal models using dosages that are higher than that traditionally needed to treat river blindness, and to define the synergistic drug profiles for possible applications in combinatorial TB therapies.

ACKNOWLEDGMENTS

We thank Takeshi Murakami for his mentorship and wise insights, Keerat Sidhu for technical support, Gaye Sweet for lab management and comments on the manuscript, D. Alland (University of Medicine and Dentistry of New Jersey), M. Larsen (Albert Einstein College of Medicine), A. W. Sturm (University of KwaZulu Natal, South Africa), and Patrick Tang (BC Centre for Disease Control) for the generous gifts of clinical isolates, and Julian Davies, Lindsay Eltis, Robert Hancock, and Richard Stokes for other bacterial strains. We also thank the University of British Columbia (UBC) Facility for Infectious Disease and Epidemic Research (FINDER) for technical support. Assistance from the Centre for Drug Research and Development is gratefully acknowledged.

L.E.L. was supported by a scholarship from the Canadian Natural Sciences and Engineering Research Council. This work was supported by grants from The Canadian Institute of Health Research (MOP-82745 and MOP-82855), the British Columbia Lung Association (C.J.T.), Grand Challenges Canada—Stars in Global Health (0030-01-04-01-01) (S.R.-G.), and the National Institutes of Health (AI26170 and CFAR AI-051519) (W.R.J.).

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5
THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 8, pp. 6233–6240, February 25, 2011
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Small Molecule Inhibitors That Selectively Block Dengue Virus Methyltransferase*[S]

Received for publication, August 26, 2010, and in revised form, November 23, 2010 Published, JBC Papers in Press, December 8, 2010, DOI 10.1074/jbc.M110.179184

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Crystal structure analysis of Flavivirus methyltransferases uncovered a flavivirus-conserved cavity located next to the binding site for its cofactor, *S*-adenosyl-methionine (SAM). Chemical derivatization of *S*-adenosyl-homocysteine (SAH), the product inhibitor of the methylation reaction, with substituents that extend into the identified cavity, generated inhibitors that showed improved and selective activity against dengue virus methyltransferase (MTase), but not related human enzymes. Crystal structure of dengue virus MTase with a bound SAH derivative revealed that its N6-substituent bound in this cavity and induced conformation changes in residues lining the pocket. These findings demonstrate that one of the major hurdles for the development of methyltransferase-based therapeutics, namely selectivity for disease-related methyltransferases, can be overcome.

Methyltransferases (MTases)[3] play key roles in normal physiology and human diseases through methylating DNA, RNA, and proteins. Almost all MTases use *S*-adenosyl-L-methionine (SAM) as a methyl donor and generate *S*-adenosyl-L-homocysteine (SAH) as a by-product. Pharmacological modulation of MTases by small molecules represents a novel approach to therapeutic intervention in cancer and other diseases (1). However, because the core domains of various MTases are conserved, designing inhibitors that specifically block the disease-related MTase without affecting other MTases, has been challenging. The ability to rationally design and generate selective inhibitors would have profound implications for development of new medicines for many methyltransferase-mediated diseases.

* This work was supported in part by BMRC Grant 06/1/22/19/447 (to J. L.).
The atomic coordinates and structure factors (codes 3P8Z and 3P97) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.
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[3] The abbreviations used are: MTase, methyltransferase; SAM, *S*-adenosyl-methionine; SAH, *S*-adenosyl-homocysteine; DENV, Dengue virus; hRNMT, human RNA MTase; RMSD, root-mean-square deviation; SAR, structure-activity relationship.

Dengue virus (DENV), from genus Flavivirus in the family Flaviviridae, is the most prevalent mosquito-borne viral pathogen that infects humans. The four serotypes of DENV (DENV-1 to -4) pose a public health threat to 2.5 billion people worldwide, and cause 50–100 million human infections each year. Neither vaccine nor antiviral therapy is currently available for DENV. The flavivirus MTase methylates the guanine N7 and ribose 2′-*O* positions of the viral RNA cap in a sequential manner (*i.e.* GpppA-RNA → m7GpppA-RNA → m7GpppAm-RNA) (2, 3). Recent studies have shown that flavivirus MTase is critical for viral replication and, therefore, represents a valid target for antiviral therapeutics (4–6). We therefore examined the feasibility to design inhibitors that specifically modulate flavivirus MTase.

EXPERIMENTAL PROCEDURES

Preparation of DENV-3 MTases—The DNA fragment representing the MTase domain of DENV-3 was cloned into expression vector pGEX4T1 (Amersham Biosciences). Ala-substitution mutant MTases were prepared using a standard overlapping PCR procedure. Recombinant MTases, containing an N-terminal GST, were expressed in *Escherichia coli*. BL21 cells and purified through a GSTPrep™ FF 16/10 column (GE Healthcare). The GST tag was then cleaved by thrombin and removed from the MTases using the GST column. The MTases were further purified through gel filtration to ensure protein purity was >95%. The proteins were then concentrated using Amicon Ultra tube (MW cut-off of 10 kDa; Millipore).

Construction and Analysis of Mutant DENV-2—DENV-2 (strain TSV01) genome-length cDNA clones with specific mutations were constructed by using an infectious cDNA clone pACYC FLTSV. The methods for mutagenesis of the cDNA clone, *in vitro* transcription, RNA transfection, immunofluorescence assay, and plaque assay were reported previously (7).

Preparation of Human RNA MTase (hRNMT)—The 1st strand cDNA of hRNMT was synthesized using primer hRNMT-RT-REV (supplemental Table S1) and cellular RNA extracted from HuH7 cells. The cDNA encoding hRNMT amino acids 1–476 was PCR-amplified using primers hRNMT-Xho-FOR and hRNMT-Bam-REV (supplemental Table S1) from the RT reaction. The PCR product was cloned into expression vector pET15b (Novagen). The hRNMT pro-

FOI 26-3132 - Document 5

tein, with an N-terminal His tag, was expressed in BL21 cells, and purified through a 5-ml HiTrap-chelating HP column (Amersham Biosciences).

DENV MTase Assays—Scintillation proximity-based N-7 and 2′-*O* methylation assays were used to determine the inhibitory activities of compounds, as previously reported (8, 9). The standard deviation is calculated by the non-biased *n*-1 method: Standard deviation (S.D.) = $\sqrt{((n\Sigma\times^2 - (\Sigma(\times)^2)/(n(n-1)))}$. Nonlinear regression (curve fit) and the equation below for sigmoidal dose-response (variable slope) from GraphPad Prism version 3.02 (GraphPad Prism, Inc.) were used to interpolate values for IC_{50} values in Equation 1,

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(\{LogEC50 - X\}*Hill\ Slope)}} \quad \text{(Eq. 1)}$$

where *X* is the logarithm of concentration. *Y* is the response. *Y* starts at *Bottom* and goes to *Top* with a sigmoid shape. This is identical to the "four parameter logistic equation" (10). For determination of K_i values, the Cheng-Prusoff equation (11) shown as Equation 2 was used.

$$K_i = \frac{IC_{50}}{1 + S/K_m} \quad \text{(Eq. 2)}$$

Human DNA MTase 1A (hDNMT1A) Assay—The hDNMT1A reaction contained 2 units of hDNMT1A MTase (New England BioLabs), 1 pmol annealed double-stranded hemi-DNA oligonucleotide (supplemental Table S1), 320 nM [^{3}H-*methyl*]SAM in the hDNMT buffer (50 mM Tris/HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 5% glycerol, 100 μg/ml BSA, and 0.05% CHAPS). The reaction was assembled in a 96-well ½ area, white opaque plates (Corning). After incubation at 37 °C for 40 min, the reaction was stopped with 2× stop solution (containing streptavidin-SPA beads), and measured for [^{3}H]methyl incorporation. Dose-responsive experiments were performed to determine the K_i values of inhibitors. The double-stranded hemi-DNA oligonucleotide was prepared by annealing 40-μM hDNMT sense and antisense primers (supplemental Table S1) in 1× assay buffer through denaturing at 95 °C for 3 min and cooling to room temperature.

Human RNA guanine-7-MTase (hRNMT) Assay—The hRNMT reaction contained 40 nM hRNMT MTase, 1 pmole RNA substrate (5′-GpppAGAACCUG-biotin-TEG-3 (TriLink BioTechnologies), 640 nM [^{3}H-*methyl*]SAM in the hRNMT buffer (50 mM Tris/HCl, pH 7.5, 5 mM DTT, and 0.05% CHAPS). The RNA substrate and enzyme were first mixed in buffer in a single well in a 96-well ½ area, white opaque plates (Corning), followed by initiation of the reaction with [^{3}H-*methyl*]SAM. The reaction was carried out at 22 °C for 10 min, stopped with 2× stop solution (containing streptavidin-SPA beads), and quantified for [^{3}H]methyl incorporation.

Crystallization and Structure Determination—DENV-3 MTase was crystallized using the hanging-drop vapor diffusion method. 1 μl of reservoir solution (containing 22.5% PEG 8000, 0.2 M NaCl, 0.1 M Tris, pH 8.5, and 20 mM tri-sodium citrate) was mixed with an equal volume of MTase solution at 10 mg/ml. The unit cell parameters are given in Table 1. The asymmetric unit contains two MTase molecules. For the structure with compound, 4 mM of compound was first added to the protein solution from a stock of 50 mM (dissolved in 90% DMSO) prior to mixing with the reservoir solution. Crystals appeared overnight and grew to maximum size within a week. Crystals were frozen by transferring stepwise into a mother liquor containing an additional 10%, then 20% (v/v) glycerol as cryoprotectant, and frozen in liquid nitrogen. Diffraction data were collected at the PXII (X10SA) beam line of the Swiss Light Source, Paul Scherrer Institut, Switzerland. Indexing and scaling were performed using MOSFLM (12) and SCALA, part of the CCP4i suite (13). The structures were determined by molecular replacement using MOLREP from the CCP4i package using DENV2 MTase (PDB code 1L9K) (2) as a search probe. For the compound 10* structure, only one of the two molecules (molecule A) is bound to the inhibitor, while the other molecule C binds to the co-purifying SAH cofactor (Table 2). The bond restraints for the compound were built using SKETCHER from the CCP4i package. Manual rebuilding of the model was performed using COOT (14) and the structures were refined using REFMAC5. The quality of the structures was analyzed using PROCHECK (15).

Binding of RNA to DENV MTase Measured by Competitive Fluorescence Polarization—To measure the displacement of GTP-bodipy binding to DENV MTase by RNA, we adapted the method used by (17). Briefly, 100 nM GTP-bodipy and 300 nM MTase in 50 mM Tris/HCl, pH 7.5 plus 20 mM NaCl were incubated with 2-fold serial dilutions of RNA ranging from 0–3 μM in black 96-well ½ area microplate (Corning). Fluorescence polarization was measured using Synergy 4 microplate reader (Biotek) with λ_{ex} = 485 nm and λ_{em} = 528 nm. IC_{50} (concentration of ligand needed to displace 50% of GTP-bodipy) was determined by fitting the dose response curve with Equation 3,

$$FP = FP_0 + \frac{(FP_T - FP_0)}{1 + 10^{n(\log IC_{50} - \log[L])}} \quad \text{(Eq. 3)}$$

where FP_0 is the fluorescence polarization units (mP) obtained when no GTP-bodipy in bound and FP_T the fluorescence polarization units (mP) when all GTP-bodipy is bound. [L] is the ligand concentration and *n* represents the Hill slope obtained.

K_i for ligand was defined by the Cheng-Prusoff equation (11) shown in Equation 4,

$$K_i = \frac{IC_{50}}{1 + [GTP\text{-}bodipy]/K_d} \quad \text{(Eq. 4)}$$

where K_d is the dissociation constant of GTP-bodipy for MTase (K_d = 0.8 ± 0.2 μM).

Thermo-denaturation Assay—Three μM of MTase were incubated with 0.01X SYPRO® Orange protein gel stain (Invitrogen) in the absence or presence of 50 μM sinefungin (SF) in 1× assay buffer (50 mM Bis/Tris-HCl, pH 7.5 and 20 mM NaCl) for 10 min in a 96-well PCR white plate (Bio-Rad). The plate was then sealed with Microseal®B Adhesive sealer (Bio-Rad) and heated from 25 to 85 °C with increments of 0.5 °C using iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad). Excitation and emission wavelengths were 485 nm

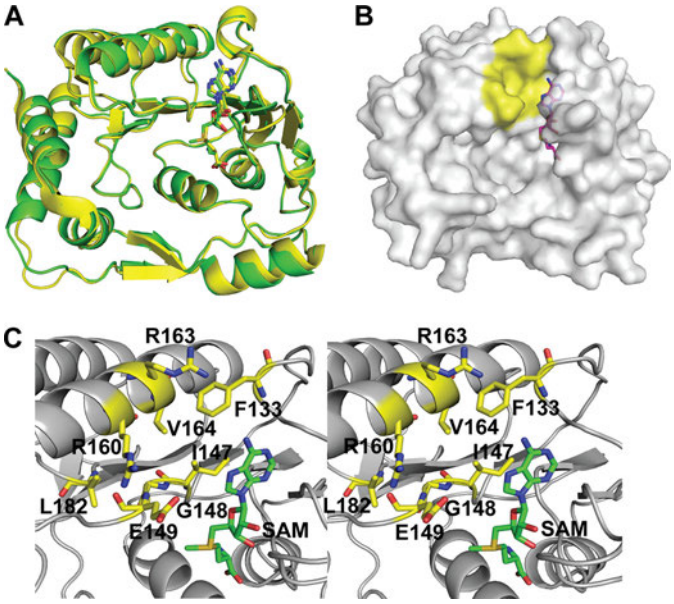


FIGURE 1. **A cavity in DENV MTase.** *A*, superposition of the crystal structures of the DENV-3 MTase-SAM complex (*yellow*) and the DENV-2 MTase-SAH complex (*green*; PDB code: 1L9K (2)). SAM and SAH are shown in *stick representation*. *B*, surface representation of the DENV-3 MTase. The additional cavity adjacent to the adenine of the SAM molecule is colored in *yellow*. The MTase protein was set to partial transparency to view the complete molecule of SAM. SAM is shown as *stick representation*. *C*, stereo view of amino acids involved in the formation of the additional cavity. Residues and the SAM molecule are labeled and shown in *stick representation*. The images were produced using PyMOL.

TABLE 1
Data collection and phasing statistics

	SAM	Compound 10*
Space group	P21212	P21212
Cell parameters (a, b, c; Å)	51.82, 61.10, 186.04	51.81, 61.10, 184.68
Wavelength (Å)	1.0	1.0
Resolution range (Å)	51.85–1.7	51.78–1.7
No. of observed reflections	455628	349066
No. of unique reflections[a]	66086 (9512)	64661 (9441)
Completeness (%)	100 (100)	98.9 (99.8)
Multiplicity	6.9 (6.9)	5.4 (5.3)
R_{merge}[b]	0.076 (0.536)	0.071 (0.424)
$I/\sigma(I)$	16.1 (3.4)	14.6 (3.2)
Solvent content (%)	42.8	41.5

[a] The numbers in parentheses refer to the last (highest) resolution shell.
[b] $R_{merge} = \Sigma_h\Sigma_i|I_{hi} - <I_h>|/\Sigma_{h,i}\, I_{hi}$, where I_{hi} is the *i*th observation of the reflection h, while $<I_h>$ is its mean intensity.

and 625 nm, respectively. MTase melting temperature (T_m) in the presence or absence of SF was determined from the graph of -dRFU/dT *versus* temperature, where RFU is relative fluorescence unit, and T is the temperature in degree Celsius.

RESULTS

DENV MTase-SAM Co-crystal Complex—We determined the crystal structure of DENV-3 MTase (representing the N-terminal 272 amino acids of the viral NS5 protein) in complex with methyl donor SAM at a resolution of 1.7 Å (PDB code 3P8Z), with a final R_{factor} of 19.2% and an R_{free} of 22% (Fig. 1; Tables 1 and 2). The structure of the SAM-MTase complex is almost identical to that of the SAH-MTase complex (2), with an overall Cα root-mean-square deviation (RMSD) of 0.49 Å (Fig. 1*A*). The current structure also closely resembles MTase structures from other members of the Flavivirus family (reviewed in Ref. 18). All residues forming the SAM/SAH-binding pocket are well superimposed (data not shown), indicating that the extra methyl group in the SAM molecule does not affect the ligand-protein interaction. Because no SAM was present during the crystallization, its presence in the structure must have originated from *E. coli* during protein expression. Biochemical analysis of the purified MTase also suggested that the SAM molecule was co-purified with the protein (supplemental Fig. S1).

TABLE 2
Refinement statistics

	SAM	Compound 10*
Resolution range (Å)	30–1.7	30–1.7
Completeness (%)	100	98.57
No of reflections		
Used for refinement	62658	61298
Used for R_{free} calculation	3345	3279
No of non hydrogen atoms (Average B factors, Å2)		
Protein chain A	2051 (18.6)	2074 (19.3)
Protein chain C	2062 (18.0)	2076 (19.9)
Compound	–	34 (16.4)
SAM (chain B; D)	27 (14.1); 27 (14.8)	–
SAH	–	26 (20.7)
Water molecules	689 (32.8)	718 (33.3)
R_{factor}[a] (%)	0.192	0.197
R_{free}[b](%)	0.220	0.231
RMS deviations from ideality		
Bond lengths (Å)	0.013	0.014
Bond angles (°)	1.64	1.57
Ramanchandran plot		
Residues in most favoured regions (%)	92.9	93.8
Residues in additional allowed regions (%)	6.7	5.7
Residues in generously allowed regions (%)	0.0	0.0
Residues in disallowed regions (%)	0.5	0.5
PDB code	3P8Z	3P97

[a] $R_{factor} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$.
[b] R_{free} was calculated with 5% of reflections excluded from the refinement.

Mutation of Residues Lining the Hydrophobic Pocket Affect Virus Replication—Structure examination located the additional hydrophobic cavity (colored in *yellow*, Fig. 1*B*) above the adenine base that was previously identified in West Nile virus (WNV) MTase (19). As shown in Fig. 1*C*, the cavity is lined by the conserved amino acids Phe-133, Ile-147, Gly-148, Glu-149, Arg-160, Arg-163, Val-164, and Leu-182, among which Phe-133 and Ile-147 also contribute to the SAM-binding pocket. Functional analysis of the identified cavity was performed through Ala mutations of these residues in the context of genome-length RNA of DENV-2. After transfection into BHK-21 cells, wild-type (WT), and mutant RNAs generated varying amounts of envelope protein-expressing cells in the order of WT>F133A≈L182A >G148A>R163A>R160A (Fig. 2*A*). All RNAs produced infectious viruses with plaque sizes of WT≈F133A≈L182A>G148A≈R163A>R160A (Fig. 2*B*). Sequencing analysis showed that all mutant viruses retained the engineered changes with no other mutations (data not shown). The growth kinetics of F133A and L182A viruses was similar to that of WT virus, whereas the G148A, R160A, and R163A viruses replicated slower than the WT virus (Fig. 2*C*). At 24 h post-infection, the viral titers of these latter three mutant viruses were about 1,000-fold less than that of WT virus (Fig. 2*C*). Similar results were obtained

FOI 26-3132 - Document 5

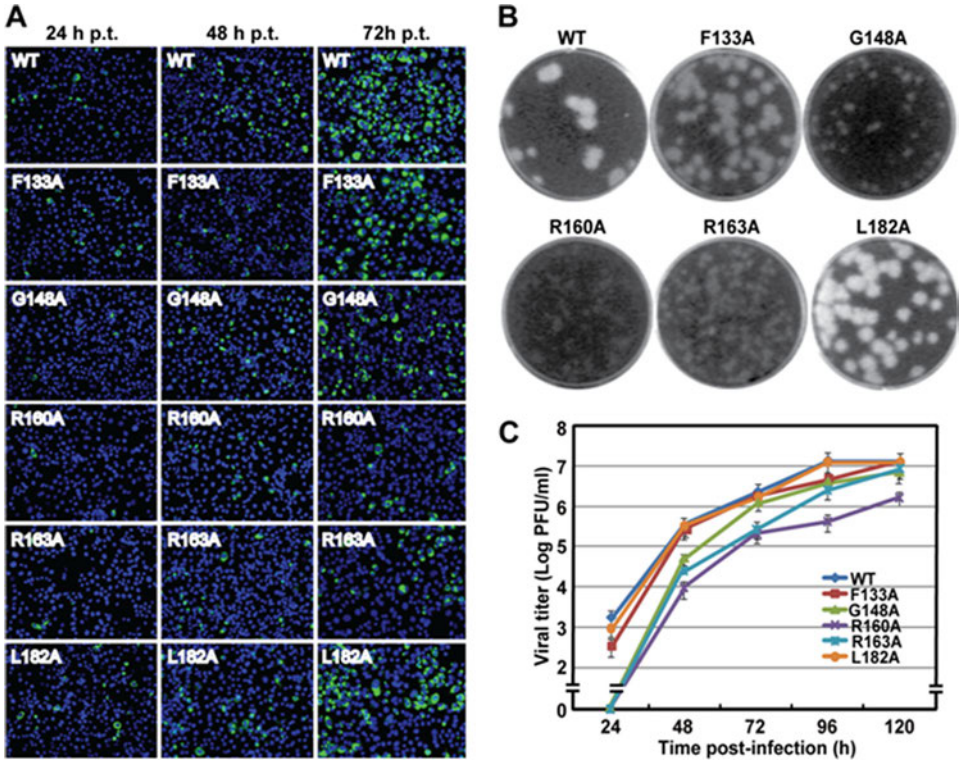


FIGURE 2. **Functional analysis of the identified cavity in DENV-2 replication.** *A*, immunofluorescence analysis (*IFA*). BHK-21 cells were electroporated with WT and mutant genome-length RNAs. The mutant genome-length RNAs contained Ala substitution at indicated positions. At various time points post-transfection (p.t.), the cells were subjected to IFA using mouse antibody 4G2 (against DENV E protein) and anti-mouse immunoglobulin G conjugated with FITC (*green*) as the primary and secondary antibodies, respectively. Nuclei were counterstained with DAPI in *blue*. *B*, plaque morphology of WT and mutant viruses from supernatants collected from Vero cells after 5 days post-infection. *C*, growth kinetics WT and mutant viruses. Vero cells were infected with the indicated viruses at an MOI of 0.1. Culture fluids of the infected cells were determined for viral titers using plaque assay. Note the scale of viral titer from 0 to 10^2 PFU/ml is different from the scale of 10^2 to 10^8 PFU/ml.

from three independent transfection experiments. The negative effect of the R160A or R163A substitution on viral replication was further confirmed using a luciferase-reporting replicon (supplemental Fig. S2). *In vitro* enzymatic assays using recombinant MTases (supplemental Fig. S3) showed that most of the mutations reduced either N-7 (R160A, R163A) or 2′-*O* (F133A, L182A) methylation activities, while both activities were significantly decreased in mutant G148A (Table 3). Thermo-denaturation analysis suggests that the defects in cap methylations are not due to misfolding of the mutant MTases as their melting temperatures are not significantly reduced compared with the WT protein. In addition, their ability to bind RNA and sinefungin was not markedly different from WT protein (Table 3). Overall, the data support results obtained with WNV that the identified cavity is important for Flavivirus cap methylations as well as replication in cells (19).

Design and Synthesis of Selective Inhibitors of Flavivirus MTase—The structural and functional results prompted us to design inhibitors that can specifically bind to the identified cavity of the flavivirus MTase. Because this cavity connects to the SAM-binding pocket at the adenine-holding site, we postulated that substituents extending from the adenine base of the SAH molecule would interact with the identified cavity. Such SAH derivatives would have the potential to selectively inhibit DENV MTase without suppressing host MTases. To test this hypothesis, we synthesized a series of SAH analogs, each with a substitution at the N-6 position of the adenine base (Fig. 3). This N6 position was selected for two reasons: (i) It is in the most optimal geometrical position for making extensions to occupy the hydrophobic cavity, and (ii) the N6 is the most chemically tractable group in the adenine ring for modifications. Compound synthesis was detailed in supplemental materials. The compounds were assayed as inhibitors of N7 and 2′-*O* methyltransferase activities using DENV-3

TABLE 3
Effects of Ala substitutions of DENV MTase hydrophobic pocket residues on DENV-3 MTase activities

DENV-3 MTase	N7 activity[a]	2′-O activity[a]	MTase melting temperature, T_m (°C) [+sinefungin, °C][b]	RNA binding[c], K_i
	%	%		μM
F133A	100 ± 1.2	65 ± 3.2	38 [+1.5]	0.017
G148A	33 ± 4.5	40 ± 5.3	35 [+1]	0.033
R160A	63 ± 4.47	102 ± 5.0	40 [+4]	0.031
R163A	59 ± 4.8	100 ± 5.1	40 [+4]	0.021
L182A	95 ± 5.9	18 ± 5.0	37.5 [+1]	0.052
WT	100	100	40 [+4]	0.048

[a] Recombinant WT and mutant MTases were assayed for N7 and 2′-*O* methyltransferase activities by quantifying the conversion of GpppA-RNA → m7GpppA-RNA and m7GpppA-RNA → m7GpppAm-RNA, respectively (4, 8). Methylation efficiencies of mutant MTases were compared with that of the WT MTase (set at 100%). An average of three experiments is shown.

[b] The stabilities of the mutant and WT proteins were assessed by determination of their melting temperature, and their abilities to bind the SAM mimetic, sinefungin, and RNA substrate. Protein melting temperatures were obtained in thermo-denaturation assays in the presence or absence of 50 μM sinefungin containing SYPRO® Orange protein gel stain (10).

[c] RNA binding was measured with a competitive fluorescence polarization assay with GTP-bodipy and GTP capped RNA-211nt corresponding to DENV 5′-UTR (8, 16).

FOI 26-3132 - Document 5

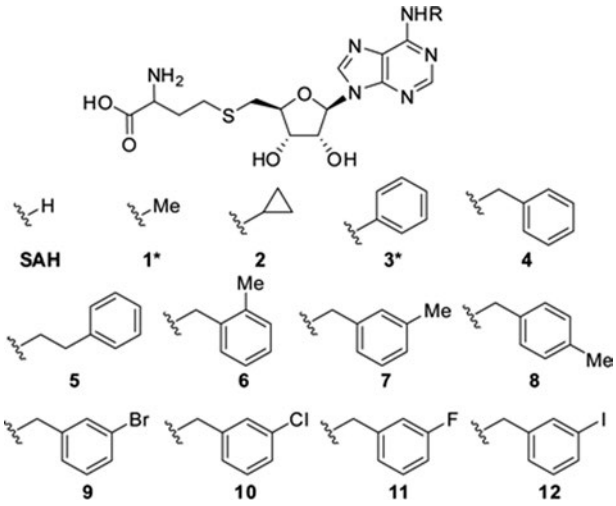


FIGURE 3. **Structures of SAH derivatives that inhibit DENV-3 MTase.**

TABLE 4

***K*$_i$ values of compound**

Each compound was tested for its inhibition against DENV-3 MTase (for both N7 and 2′-*O* MTase activities), human RNA guanine-7-MTase (hRNMT), and human DNA MTase1A (hDNMT). Symbol * indicates that the compounds are enantiopure. Average results from three independent experiments are presented.

Compound	DENV-3 MTase K_i		hRNMT K_i	hDNMT K_i
	N7	2′-*O*		
	μM	μM	μM	μM
SAH	3.19 ± 0.04	0.57 ± 0.04	4.54 ± 0.02	6.18 ± 0.02
1*	5.91 ± 0.01	1.51 ± 0.04	1.09 ± 0.05	2.75 ± 0.04
2	21.94 ± 0.05	2.07 ± 0.05	14.73 ± 0.01	2.06 ± 0.02
3*	10.86 ± 0.07	3.45 ± 0.06	16.09 ± 0.04	1.90 ± 0.03
4	2.1 ± 0.05	0.52 ± 0.05	20.85 ± 0.06	>50
5	8.26 ± 0.06	2.1 ± 0.03	26.18 ± 0.05	11.85 ± 0.02
6	6.03 ± 0.06	4.23 ± 0.07	>50	>50
7	0.85 ± 0.04	0.28 ± 0.03	>50	>50
8	1.76 ± 0.05	0.75 ± 0.05	>50	>50
9	2.15 ± 0.03	0.15 ± 0.04	>50	>50
10	0.82 ± 0.06	0.17 ± 0.02	>50	>50
11	0.77 ± 0.04	0.19 ± 0.03	>50	25.07 ± 0.10
12	1.30 ± 0.04	0.20 ± 0.06	>50	17.60 ± 0.04

MTase. For ease of synthesis, initial structure-activity relationship (SAR) was assessed with compounds synthesized as epimeric mixtures, except for compounds 1* and 3* (Symbol * indicates enantiopure; Table 4). The SAR showed that, compared with SAH, a substitution with small alkyl groups (compounds 1* and 2) or a phenyl ring (compound 3*) reduced the anti-MTase activity. Further extension of the substituent showed that a benzyl group was optimal (compound 4), retaining the activity of SAH. Lengthening the spacer by one carbon between the phenyl ring and the adenine N6 reduced the activity (compound 5). Addition of a methyl group on the aryl ring at different positions indicated that the meta position is optimal, leading to an increase in potency of almost 4-fold over the starting point (compare compound 7 with 6 and 8). Next, halogen substituents were introduced at the meta position (compounds 9–12); these compounds exhibited potencies similar to the methyl substituent analog (compound 7). Compared with the SAH starting point, compounds 7 and 9–12 consistently showed better potencies; this conclusion was further supported by the results from enantiopure compounds 10* and SAH* (Table 5). For each compound, the K_i value for N-7 methylation was higher than that for 2′-*O* methylation; this difference could result from the distinct mechanisms of the two cap methylations (20, 21). The parent compound, SAH*, also displayed similar differences in N7 and 2′-*O* inhibition. Nonetheless, the general SAR trend was the same in N-7 and 2′-*O* methyltransferase activities. In stark contrast to SAH and compounds 1–3, the compounds with extended phenyl rings (compounds 4 and 5), and N-6 benzylic substituents (compounds 6–12) had either partially or completely lost the activity against human RNA guanine-7-MTase (hRNMT), DNA MTase 1A (hDNMT1A; Tables 4 and 5), and SET7/9 histone methyltransferases (hHMT; data not shown). These results demonstrate that the N6 modified SAH derivatives can selectively inhibit DENV MTase.

TABLE 5

***K*$_i$ values of SAH* and compound 10* in various MTases**

Symbol * indicates that the compounds are enantiopure. Average results from three independent experiments are presented.

MTase	Activity	SAH* K_i	10* K_i
		μM	μM
DENV-3 WT	N7	0.65 ± 0.03	0.24 ± 0.07
	2′-*O*	0.28 ± 0.04	0.08 ± 0.01
DENV-3 F133A	N7	1.16 ± 0.02	0.0020 ± 0.0002
	2′-*O*	0.095 ± 0.010	0.007 ± 0.002
WNV WT	N7	2.44 ± 0.06	5.68 ± 0.05
	2′-*O*	0.020 ± 0.003	0.044 ± 0.010
hRNMT	N7	0.38 ± 0.05	>50
hDNMT	C5	1.38 ± 0.02	>50

Because the identified cavity is conserved among flavivirus MTases, we examined whether the SAH analogs would inhibit flavivirus MTase other than DENV. As shown in Table 5, compound 10* also inhibited WNV MTase but was about 2.2-fold less potent than SAH*. Nevertheless, compound 10* did not inhibit host hRNMT or hDNMT (Table 5), indicating its selectivity for flavivirus MTase.

Crystal Structure of DENV-3 MTase Compound Complex—To provide structural evidence for mode-of-action, we solved the crystal structure of DENV-3 MTase in complex with compound 10* at a resolution of 1.7 Å (PDB code 3P97; Tables 1 and 2). The overall structure of the compound 10*-MTase complex is almost identical to that of the SAM-MTase complex, with a Cα RMSD of 0.16 Å (Fig. 4*A*). As shown in Fig. 4, *A* and *B*, the N6 benzyl ring of compound 10* fits snugly into the identified cavity without changing the binding of the SAH moiety. Remarkably, the benzyl ring of the compound induces a local conformational change within the cavity (Fig. 4, *C* and *D*): the side chains of Phe-133 flips away from the cavity by 100 degrees (measured at the Cα and Cβ torsion angle) to create space for the N6 benzylic ring, and the side chains of Arg-163 shifted by 3.5 Å (measured at the NH1 atom) to stack onto the benzylic ring, resulting in the formation of a cation-π interaction. Fig. 4*E* summarizes the interactions established between compound 10* and the DENV-3 MTase.

Effect of Compound-induced Conformational Change on Efficacy—The observed flipping of Phe-133 in the co-crystal structure of DENV-3 MTase-compound complex suggests that this residue at its native conformation sterically blocks the binding of the N6 benzylic ring. If this is the case, substitution of Phe-133 to a residue with a smaller side chain would facilitate the binding of the benzylic ring of compound 10*;

FOI 26-3132 - Document 5

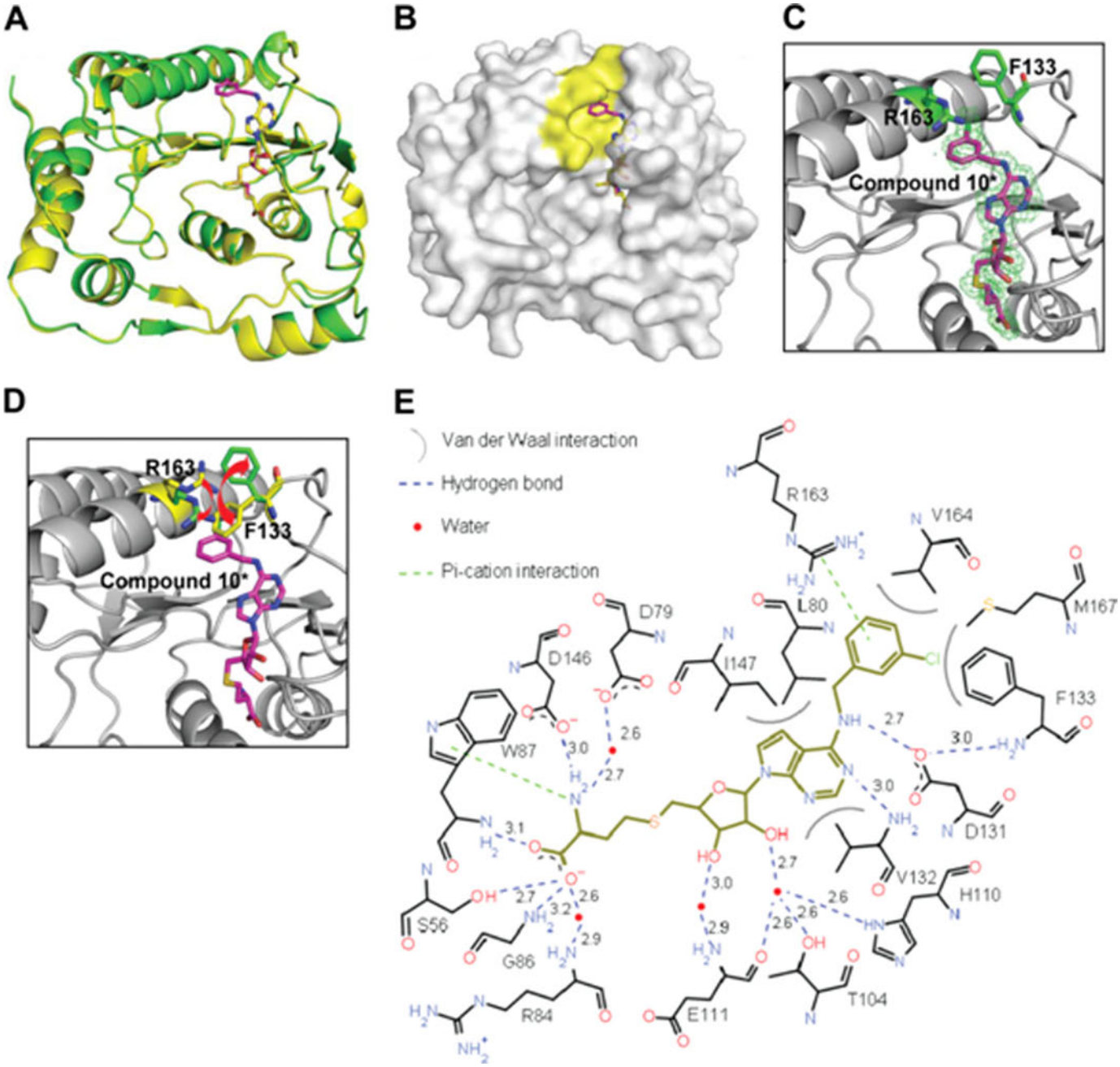


FIGURE 4. **Co-crystal structures of DENV-3 MTase in complex with compound 10*.** *A*, superposition of the crystal structures of compound 10*-DENV-3 MTase (*green*) complex and the MTase-SAM complex (*yellow*). SAM is shown in *stick representation*. Compound 10* is colored in *magenta*. *B*, surface representation of the DENV-3 MTase, showing the binding of compound 10* (in *stick presentation*) in the identified cavity. *C*, representative omit ($F_o - F_c$) electron density map (*green*) at a level of 2.5 σ showing the bound compound 10* molecule. Amino acids Phe-133 and Arg-163 are labeled. *D*, compound-induced conformational change of amino acids. Compound 10* is shown in stick presentation (*magenta*). Residues Phe-133 and Arg-163 in the SAM-MTase complex are colored in *yellow*; the same residues in the compound 10*-MTase complex are shown in *green*; two arrows (*red*) indicate the compound-induced conformational change. *E*, schematic view of the interactions between compound 10* (*brown*) and the DENV-3 MTase (*black*).

consequently, the mutant MTase would be more sensitive than the WT MTase to compound inhibition. To test this hypothesis, we prepared a mutant MTase containing a Phe → Ala substitution at amino acid position 133. Indeed, the K_i values of the F133A MTase were lower (120-fold for N7 activity and 11-fold for 2′-*O* activity) than that of the WT MTase (Table 5). These results demonstrate that the selective inhibition of compound 10* against DENV MTase is obtained through binding of the N6 benzylic ring to the identified pocket.

DISCUSSION

We used DENV MTase as a model to prove the concept that selective inhibitors of disease-related MTase could be obtained. The selectivity was accomplished through designing SAH derivatives with substituents in the adenine moiety that

FOI 26-3132 - Document 5

extend into a cavity located above the SAM-binding pocket. The newly identified pocket is unique to the flavivirus MTases (18, 19) and was not found in the crystal structures of several human MTases available in the Protein Data Bank (data not shown). The starting compound, SAH, is a well-known product inhibitor, which is non-selective and inhibits eukaryotic, bacterial, and viral MTases (22–24). In our hands, it showed similar inhibitory properties against DENV, WNV, human RNA, DNA, and histone MTases (data not shown). Attachment of small alky or phenyl groups to the N6-position in its adenine base failed to improve its potency against DENV MTase or hRNMT, but moderately enhanced its activity against hDNMT by 2–3-fold. Our findings contradicts a previous report in which compound 3 was found to be inactive against hDNMT (25). Introduction of a benzyl ring retained its activity against DENV MTase and strongly reduced its activity against hRNMT. No activity was observed against hDNMT. Thus, some gain in selectivity for DENV MTase was possible through this modification. This result is in agreement with the hDNMT data obtained by Saavedra *et al.* (25). Extension of the benzyl group with a second carbon chain significantly reduced inhibition against all enzymes tested. It is possible that the hydrophobic pocket does not accommodate this longer extension; alternatively, the loss of activity may be due to greater flexibility of the carbon linker and resultant poorer binding into the cavity. Further probing with substituents in the aryl ring revealed that additions in the meta-position provided moderate improved potency for DENV MTase but gained selectivity against hRNMT, DNMT and histone MTases (data not shown). Substitution with either a methyl-, chloro- or fluoro- group in the meta-position resulted in about 4-fold better inhibitory properties compared with SAH. Replacement of a bromo- or iodo-group in this position exhibited similar inhibitory properties on DENV MTase, as the un-substituted aryl ring, indicating that bulky groups on the aryl ring were not preferred. On the other hand, substitution in the ortho position was not advantageous, while the para position conferred some improvement against DENV N7 activity only. Remarkably, additions in all three positions rendered the compounds either inactive or poorly active against hRNMT, hDNMT, and hHMT (data not shown).

Residue Phe-133 is conserved in the several mosquito- and tick-borne Flaviviruses, including DENV, WNV, and JEV (19). Mutation of this amino acid to Ala did not affect DENV N7 and 2′-*O* activity *in vitro*, nor impaired DENV growth in cells. Thus, this residue is not critical for virus replication. Furthermore, Phe-133 is rather flexible, as binding of compound 10* in DENV MTase induced its side chain to rotate away from the cavity, to accommodate its N6-substituted aryl ring. It is possible that other residues lining the pocket may have similar mobilities. Indeed, the improved potency of compound 10* over SAH* can be partially explained by the movement of residue Arg-163, to form a strong cation-π interaction with the N6-aryl ring of compound 10*.

Although the benzyl-SAH derivatives were selective for both DENV and WNV MTases, confirming the importance of the hydrophobic pocket (19), the most active compound, 10*, was not more potent in WNV compared with SAH*. Detailed examination of the hydrophobic pockets in both MTases did not reveal any obvious difference between the two sites, and the reason for the discrepancy in the potency of the compounds for the two virus enzymes is unknown. Nevertheless, both SAH* and compound 10* are significantly more potent on WNV 2′-*O* activity compared with the same activity in DENV (14 and 18-fold better, respectively, in WNV). In contrast, the opposite is true for their N7 activities. Both compounds respectively, showed 4- and 24-fold less potency in WNV than DENV. Thus, the observed difference in the two viral MTases for this class of compounds may be related to variations in their N7 and 2′-*O* mechanisms (20, 21). In summary, using a structure-guided approach, we have designed DENV selective and potent inhibitors by taking advantage of a specific pocket found in Flavivirus MTases, and provided a paradigm that may facilitate pharmacological therapeutics targeting other disease-related MTases.

Acknowledgments—We thank Jan Jiricek, Cheah Chen Seh, Daying Wen, and colleagues at Novartis Institutes for BioMedical Research in Shanghai for help. We thank the beamline scientists at the Swiss Light Source for assistance.

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FOI 26-3132 - Document 5

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Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
49(5) 1–14
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Ivermectin in combination with doxycycline for treating COVID-19 symptoms: a randomized trial

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Abstract

Objective: We evaluated whether ivermectin combined with doxycycline reduced the clinical recovery time in adults with COVID-19 infection.

Methods: This was a randomized, blinded, placebo-controlled trial in patients with mild-to-moderate COVID-19 symptoms randomly assigned to treatment (n = 200) and placebo (n = 200) groups. The primary outcome was duration from treatment to clinical recovery. Secondary outcomes were disease progression and persistent COVID-19 positivity by RT-PCR.

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FOI 26-3132 - Document 5

Results: Among 556 screened patients, 400 were enrolled and 363 completed follow-up. The mean patient age was 40 years, and 59% were men. The median recovery time was 7 (4–10, treatment group) and 9 (5–12, placebo group) days (hazard ratio, 0.73; 95% confidence interval, 0.60–0.90). The number of patients with a $\leq$7-day recovery was 61% (treatment group) and 44% (placebo groups) (hazard ratio, 0.06; 95% confidence interval, 0.04–0.09). The proportion of patients who remained RT-PCR positive on day 14 and whose disease did not progress was significantly lower in the treatment group than in the placebo group.
Conclusions: Patients with mild-to-moderate COVID-19 infection treated with ivermectin plus doxycycline recovered earlier, were less likely to progress to more serious disease, and were more likely to be COVID-19 negative by RT-PCR on day 14.
Trial Registration: ClinicalTrials.gov Identifier: NCT04523831.
Data Repository ID: Dryad. doi:10.5061/dryad.qjq2bvqf6

Keywords
Ivermectin, doxycycline, COVID-19, recovery time, infection, reverse transcription polymerase chain reaction

Date received: 1 January 2021; accepted: 6 April 2021

Introduction

On 31 December 2019, the World Health Organization announced the outbreak of an atypical pneumonia in Wuhan City, China. Within weeks, the illness became a pandemic.[1,2] The presentation of the disease, named coronavirus disease 2019 (COVID-19), ranges from asymptomatic to fatal.[3] Death is mainly caused by pneumonia and potentially also by hyperinflammation associated with cytokine storm syndrome.[4] To date, treatment has relied mainly on supportive care.

Numerous clinical trials worldwide have explored the efficacy of existing medicines against COVID-19, including various antiviral and immunomodulatory drugs.[5] Australian researchers from Monash University established the efficacy of ivermectin, a broad-spectrum antiviral drug,[6,7] against severe acute respiratory syndrome-related coronavirus (SARS-CoV-2) in *in vitro* studies; viral replication was stopped within 24 to 48 hours.[8] Tetracycline, a widely available and well-tolerated antibiotic useful against atypical infections,[9] has known anti-inflammatory effects[10] and, along with its synthetic derivative, doxycycline, has been shown by Mohit et al.[11] to potentially be effective against COVID-19. The antiviral and anti-inflammatory properties of ivermectin combined with doxycycline may be beneficial in the treatment of COVID 19. Given that these two drugs have different modes of action, their synergistic effects may contain viral infection by targeting different sites of disease pathogenesis. In the present study, we sought to determine the efficacy of this combination in patients with mild-to-moderate COVID-19 symptoms.

Patients and methods

Patients

Patients were enrolled between 1 June 2020 and 30 August 2020. The inclusion criteria

FOI 26-3132 - Document 5

for enrollment were as follows: age >18 years, positive COVID-19 reverse transcription polymerase chain reaction (RT-PCR) test within 3 days prior to enrollment, and mild to moderately severe COVID-19 infection. Patients who were unable to receive oral medications, were pregnant or breastfeeding, had severe COVID-19 symptoms (defined as tachypnea [>30 breaths/minute] and hypoxia [oxygen saturation (SpO2) <90%] requiring supplemental oxygen), were admitted to intensive care or high-dependency units, or had known hypersensitivities to ivermectin or doxycycline were excluded from participation.

Trial design

This was an investigator-initiated, placebo-controlled trial. The independent data safety monitoring board of Dhaka Medical College monitored treatment safety and efficacy. Routine investigations were performed at the pathology laboratory of Dhaka Medical College. RT-PCR tests were performed at the virology laboratory of Dhaka Medical College and Bangabandhu Sheikh Mujib Medical University. Ivermectin, doxycycline, and placebo preparations were provided by Popular Pharmaceuticals Ltd. (Dhaka, Bangladesh).

Eligible patients were randomly assigned to the treatment group or the placebo group at a 1:1 ratio on day 1 of the trial. Group assignment was not stratified according to disease severity.

The allocation schedule was created with a list of random numbers generated using a random number generator program by the head of the Department of Medicine of Dhaka Medical College. Group assignment was concealed in sequentially numbered, opaque, sealed envelopes. The randomization code was maintained by the pharmaceutical company. Both the investigators and the patients were blinded to the treatment allocation. Decoding was performed at the end of the trial under the supervision of the principal of the institute. The trial was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines, and was approved by the ethical review committee of Dhaka Medical College Hospital (ERC-DMC/ECC/2020/117). Dhaka Medical College is the largest tertiary care teaching hospital and the largest COVID-19 treatment hospital in Bangladesh. The trial was registered retrospectively at ClinicalTrials.gov (identifier: NCT04523831). Written informed consent was obtained from all patients prior to participation. The study is reported according to the Equator Network Guidelines.

Study interventions

The treatment group received a single dose of ivermectin 12 mg and doxycycline 100 mg, twice daily for 5 days, in addition to standard of care. Standard of care included administration of paracetamol, antihistamines, cough suppressants, vitamins, oxygen therapy according to indication and need, low molecular weight heparin according to indication, appropriate other broad-spectrum antibiotics, remdesivir injection, other antiviral drugs, and other drugs for associated comorbid conditions.

The placebo group received placebo in addition to standard of care.

Experimental procedures

The baseline demographic and clinical characteristics of patients were recorded. The date of random assignment was considered as day 1, and all patients received their initial study intervention on day 1. Outpatients were followed daily until at least 3 days of clinical recovery were observed. Clinical recovery was defined as a normal body temperature of 36.1°C to 37.2°C maintained for at least 3 days,

FOI 26-3132 - Document 5

significantly improved respiratory symptoms (respiratory rate <25 breaths/minute, no dyspnea), and oxygen saturation greater than 93% without assisted oxygen inhalation, as recommended by the World Health Organization and the national guidelines of Bangladesh.[12,13]

Hospitalized patients were followed from day 1 through day 14 or until discharge or clinical improvement, whichever occurred later. Clinical status and vital signs (including respiratory status) were recorded daily. Adverse events as defined by the Medical Dictionary for Regulatory Activities (MedDRA) were documented.

Laboratory tests were performed on day 1, and included the following: complete blood count, random blood glucose, creatinine, alanine transaminase, C-reactive protein, ferritin, and D-dimer. Chest radiography or chest CT scanning were performed as needed. For outpatients, test results were documented at the next visit. Hospitalized patients were tested on days 1 and 7 and when ordered by the treating physicians. COVID-19 RT-PCR testing was performed 14 days after the initial positive test in all patients.

Outcome measures

The primary outcome was the number of days required for clinical recovery from day 1. Clinical recovery was divided into three categories: early recovery within 7 days, intermediate recovery within 7 to 11 days, and late improvement requiring 12 or more days for recovery. Secondary outcomes were disease progression through mild, moderate, severe, or death, and the proportion of patients who continued to test positive for COVID-19 on day 14. Adverse drug reactions (adverse events assumed to be caused by the study intervention) were also recorded. Mild disease was defined as symptoms of an upper respiratory tract viral infection, including mild fever, dry cough, sore throat, nasal congestion, headache, muscle pain, anosmia, and malaise. Moderate respiratory symptoms such as cough and shortness of breath were observed without signs of severe pneumonia. Severe disease included severe dyspnea, tachypnea (>30 breaths/minute), and hypoxia (SpO_2 <90% at room air). These classifications were according to the World Health Organization and national guidelines of Bangladesh.[12,13] The co-investigators assessed the outcome, graded the disease, and documented adverse reactions.

Statistical methods

We sought to enroll as many patients as possible in a short time. As a result, a priori power calculations were not performed. Instead, 200 patients were enrolled in each group and it was assumed that 20% of patients in each group would be lost to follow-up. A post-hoc power calculation $[2SD^2 (Z_{\alpha/2} + Z_{\beta})^2/d^2]$[14], a two-tailed test, an alpha level of 0.05, and standard deviation (SD) 6 indicated that the enrollment of 141 patients in each group (170 before 20% attrition) would result in an 80% chance of detecting a 2-day difference in median recovery time.

Intention-to-treat analysis was performed. The difference in median recovery time was determined using Kaplan–Meier analysis and a log-rank test. Details of patients who were lost to follow-up, had died, or had withdrawn from the trial owing to adverse effects were censored on the final study day. Differences in the clinical improvement in the three disease categories were analyzed using logistic regression. Hazard ratios (HRs) were calculated using Cox regression analysis. Categorical variables are presented as n (%), normally distributed continuous variables as mean (SD), and skewed continuous

FOI 26-3132 - Document 5

variables as median (interquartile range [IQR]).

Subgroup analysis was performed using the logistic regression analysis. HRs were calculated using Cox regression analysis. Age group, sex, fever, cough, respiratory distress, hypertension, diabetes, and presence of any comorbidity were adjusted. Subgroups consisted of age (less than vs. more than 60 years), sex, presence or absence of any comorbidities, mild disease, moderate disease, hospital admission, and interval between disease onset and drug application interval (less than vs. more than 4 days from the onset of the disease). A forest plot was constructed to evaluate differences in the primary outcome among the subgroups. No corrections were made for multiple comparisons. All tests were two-tailed and the alpha was set at 0.05. Data were analyzed using SPSS software, version 20 (IBM Corp., Armonk, NY, USA).

Results

Sample characteristics

Of 556 screened patients, 400 were enrolled and randomly assigned to the treatment and placebo groups (Figure 1). Among the 200 patients in the placebo group, 17 were lost to follow-up, 3 died, and 180 completed the follow-up. Among the 200 patients in the treatment group, 15 were lost to follow-up, 2 discontinued owing to adverse effects, and 183 completed follow-up. None of the patients were receiving oxygen therapy when randomized because only patients with mild and moderate symptoms were

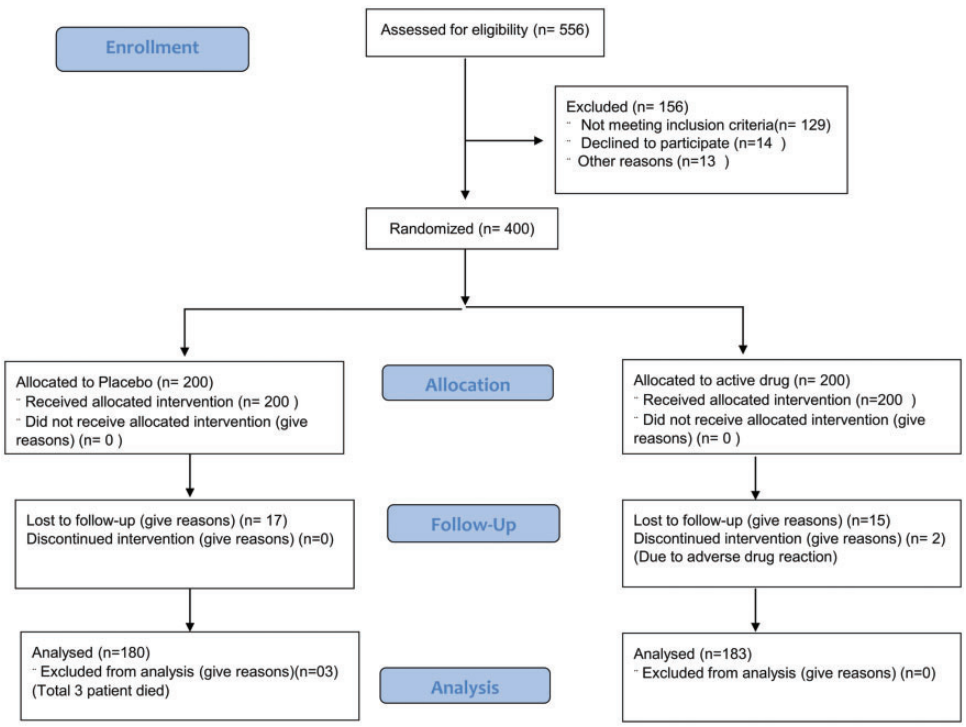


Figure 1. Enrollment, randomization, follow up, and analysis of patients according to the CONSORT 2010 flow diagram.

FOI 26-3132 - Document 5

included. A total of 132 (33%) patients required hospital admission during the study period. Among the hospitalized patients, 58% had moderate disease. The condition of 48 patients worsened during the study period: 5 patients worsened from mild disease to moderate disease but did not require oxygen and the remaining 43 patients worsened from mild/moderate to severe disease and required oxygen therapy.

Patient disposition and characteristics

The two groups were balanced in terms of demographics and disease characteristics (Table 1). The mean age of all patients was 40 years, and 59% were men. Most patients presented with fever (300, 75%) or cough (247, 62%). Respiratory distress (defined as shortness of breath and respiratory rate >25 breaths/minute) was noted in 123 (31%) patients at presentation. Furthermore, 277 patients (69%) had mild disease and 41 (10%) presented with at least one comorbidity (Table 2). Patients were randomly assigned to the trial within a median (IQR) of 4 (3–5) days of symptom onset (Table 2).

Primary outcome

The median (IQR) recovery period was 7 (4–10) days in the treatment group and 9 (5–12) days in the placebo group (hazard ratio [HR], 0.73; 95% CI, 0.60–0.90; p = 0.003 [Figure 2; Table 2]).

Secondary outcomes

The proportion of patients who recovered within 7 days of treatment initiation was higher in the treatment group than in the placebo group (111 [60%] vs. 80 [44%], HR, 0.06, 95% CI, 0.04–0.09 p = <0.001). The recovery rates between 7 and 11 days were not significantly different between the groups (treatment group, 47%; placebo group, 53%). The treatment group was significantly less likely to experience symptoms that persisted for more than 12 days (42 [23%] vs. 67 [37%], HR, 0.04, 95% CI, 0.03–0.07, p = <0.001) (Table 2). Patients in the treatment group were also less likely to experience disease progression and less likely to have a positive COVID-19 RT-PCR test result after 14 days (Table 2).

Post-hoc subgroup analysis

In the post-hoc analysis, differences in the primary outcome were determined in relation to the time interval between symptom onset and study intervention administration (<4 days vs. ≥4 days), age (<60 years vs. ≥60 years), sex, symptoms, disease severity, and presence of any comorbidities. No relationship was found between the outcome and patients who received study intervention after 4 days of symptom onset, were older than 60 years, were women, had moderately severe disease, and had comorbidities (Figure 3).

Safety outcomes

Among the 400 included patients, adverse drug reactions occurred in 9 patients (2.5%); of these, 2 patients discontinued intervention owing to erosive esophagitis. Non-ulcer dyspepsia developed in seven (1.75%) patients (Appendix 1). Three patients in the placebo group died; these patients had a higher mean age than those who survived (63 years vs. 39 years) (Appendix 2) and they died 8, 22, and 28 days after randomization of respiratory failure due to COVID-19-related pneumonia.

Discussion

In the present study, patients with mild or moderate COVID-19 infection treated with ivermectin in combination with doxycycline generally recovered 2 days earlier than

FOI 26-3132 - Document 5

Table 1. Baseline characteristics of patients with COVID-19 treated with ivermectin and doxycycline or placebo.

Characteristic	Total patients (n = 400)	Treatment group (n = 200)	Placebo group (n = 200)
Age, mean (SD), years	40 (13)	41 (14)	38 (12)
Age group, n (%)			
<40 years	248 (62)	132 (66)	116 (58)
40–60 years	122 (31)	56 (28)	66 (33)
>60 years	30 (8)	12 (6)	18 (9)
Men, n (%)	235 (59)	123 (62)	112 (56)
Time between onset of symptoms and enrollment, median (IQR), days [a]	4 (3–5)	4 (3–5)	4 (3–5)
Symptoms n (%)			
Fever	300 (75)	151 (76)	149 (75)
Cough	247 (62)	126 (53)	121 (61)
Running nose	36 (9)	17 (9)	19 (10)
Respiratory distress [b]	123 (31)	59 (30)	64 (32)
Sore throat	93 (23)	46 (23)	47 (24)
Hoarseness	5 (1)	2 (1)	3 (1.5)
Chest pain	25 (6)	10 (5)	15 (8)
Diarrhea	32 (8)	18 (11)	14 (5)
Vomiting	20 (5)	9 (5)	11 (6)
Anorexia	119 (30)	53 (27)	66 (33)
Anosmia	154 (39)	73 (37)	81 (41)
Headache	80 (20)	34 (17)	46 (23)
Lethargy	106 (27)	55 (28)	51 (25.5)
Conjunctivitis	8 (2)	4 (2)	4 (2)
Body ache	73 (18)	32 (16)	41 (20.5)
Co-morbidities [c]	41 (10)	19 (10)	22 (11)
Hypertension	57 (14)	29 (15)	28 (14)
Diabetes	53 (13)	24 (12)	29 (15)
Asthma	21 (5)	9 (5)	12(6)
Chronic kidney disease	8 (2)	3 (2)	5 (3)
Disease severity [d]			
Mild	277 (69)	141 (71)	136 (68)
Moderate	123 (31)	59 (30)	64 (32)

[a] Time from onset of first symptoms to first dose of study intervention.
[b] Shortness of breath, respiratory rate >25 breaths/minute, or oxygen saturation <93%.
[c] Presence of any co-morbidity.
[d] Disease severity at presentation. Mild disease: symptoms of an upper respiratory tract viral infection including mild fever, cough (dry), sore throat, nasal congestion, malaise, headache, muscle pain, anosmia, or malaise. Moderate disease: respiratory symptoms such as cough and shortness of breath were present without signs of severe pneumonia (tachypnea >30 breaths/minute, and hypoxia: oxygen saturation <90% at room air).
SD, standard deviation; IQR, interquartile range.

those treated with placebo. The proportion of patients responding within 7 days of treatment was significantly higher in the treatment group than in the placebo group. The proportions of patients who remained symptomatic after 12 days of illness and who experienced disease progression were significantly lower in the

FOI 26-3132 - Document 5

Table 2. Outcomes of patients with COVID-19 treated with ivermectin and doxycycline or placebo.

Parameter	Total patients Number of events = 263	Treatment group Number of events = 183	Placebo group Number of events = 180	Hazard ratio (95% CI)	*P*-value
Recovery, median (IQR), days[a]	7 (4–12)	7 (4–10)	9 (5–12)	0.73 (0.60–0.90)	0.003
Patients responding within 7 days, n (%)[b]	191 (51.9)	111 (60.7)	80 (44.4)	0.06 (0.04–0.09)	<0.001
Patients responding within 7–11 days, n (%)[b]	68 (18.5)	32 (47.1)	36 (52.9)	1.02 (0.77–1.36)	0.90
Patients remaining symptomatic after 12 days, n (%)[b]	109 (29.6)	42 (22.9)	67 (37.2)	0.04 (0.03–0.07)	<0.001
Increase in stage of severity, n (%)[c]	48 (13)	16 (8.7)	32 (17.8)	0.43 (0.38–0.62)	<0.001
Persistent COVID-19 RT-PCR positivity, n (%)[d]	50 (13.6)	14 (7.6)	36 (20)	0.61 (0.44–0.83)	0.002

[a] Clinical recovery was defined as a normal body temperature for at least 3 days, improved respiratory symptoms defined as no shortness of breath and respiratory rate <25 breaths/minute, and oxygen saturation >93% without supplemental oxygen.

[b] Response criterion was the recovery of patients as defined above. The day on which clinical recovery started was considered the response day.

[c] Disease stages were defined as follows:

Mild: symptoms of an upper respiratory tract viral infection, including a mild fever, cough (dry), sore throat, nasal congestion, malaise, headache, muscle pain, anosmia, or malaise.

Moderate: respiratory symptoms such as cough and shortness of breath were present without signs of severe pneumonia (tachypnea >30 breaths/minute, and hypoxia: oxygen saturation <90% at room air.

Severe: tachypnea >30 breaths/minute and hypoxia: oxygen saturation <90% at room air.

[d] Persistent COVID-19 RT-PCR positivity: positive COVID-19 RT-PCR test at 14 days.

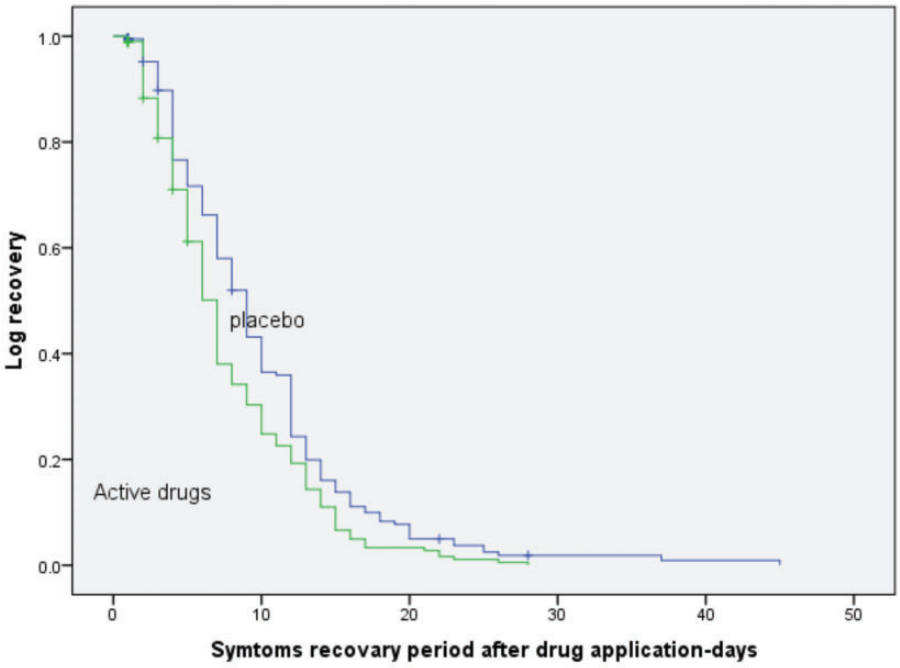
CI, confidence interval; COVID-19, coronavirus disease 2019; IQR, interquartile range; RT-PCR, reverse transcription polymerase chain reaction.

treatment group than in the placebo group. The proportion of patients who remained RT-PCR positive for COVID-19 was also lower in the treatment group than in the placebo group.

Effective vaccines and drugs for COVID-19 infection are still being researched. Potential therapies such as hydroxychloroquine[15,16] and tocilizumab[17] have to date been proved ineffective, while only remdesivir has shown some benefit in patients with moderate[18] or severe COVID-19 infection.[19] However, mortality from COVID-19 remained high, despite the use of remdesivir; therefore, treatment with an antiviral drug alone is unlikely to be sufficient. Moreover, remdesivir is expensive and can only be administered to hospitalized patients, and its effects on disease progression and viral clearance remain unclear.[18] Thus, there remains an urgent need for inexpensive and effective treatment for this highly infectious and serious disease.

We were encouraged by the in vitro research findings of researchers at Monash University, Australia;[6] the study revealed that ivermectin could reduce viral replication within 24 to 48 hours of treatment. There was a >5000-fold reduction in viral RNA with 5 μM ivermectin in cell culture, equating to a >99% reduction in viral RNA. Schmith et al.[20] stated,

FOI 26-3132 - Document 5



Footnote

	Number of patients in the analysis				
Group	Day 1	Day 10	Day 20	Day 30	Day 40
Placebo, n	200	77	13	2	1
Treatment, n	200	54	06	00	00
Placebo censored, n	15	03	00	02	00
Treatment censored, n	13	04	00	00	00

Figure 2. Time-to-recovery in the treatment and control groups, with and without censored data. Hazard ratio (95% confidence interval): 0.73 (0.60–0.90); $P = 0.003$.

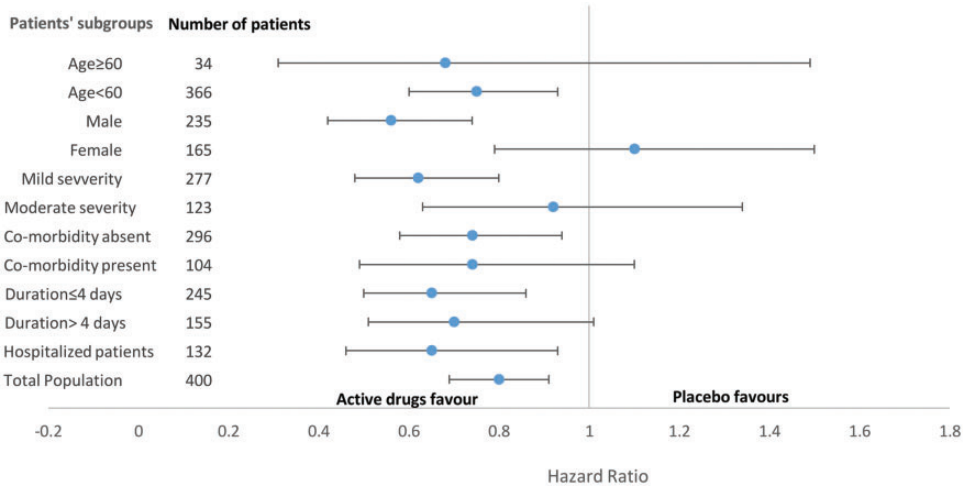


Figure 3. Post-hoc analysis of time-to-recovery among the subgroups. Data are presented as hazard ratios and 95% confidence intervals.

FOI 26-3132 - Document 5

"The concentration resulting in 50% inhibition (IC_{50}; 2 μM) was >35× higher than the maximum plasma concentration (C_{max}) after oral administration of the approved dose of ivermectin 200 μg/kg when given fasted," and predicted that with the oral dose of ivermectin 200 μg/kg, lung concentrations would be approximately one-fourth of the IC_{50}.[20] The safety of higher doses has not been evaluated in humans. Therefore, we used the conventional dose of ivermectin in the present study.

Doxycycline, which may be used for the treatment of atypical bacterial pneumonia and community-acquired pneumonia,[21] exerts an anti-inflammatory effect mediated by chelating zinc compounds on matrix metalloproteinases (MMPs) in mammalian cells.[22] Doxycycline also has antiviral activity, especially against dengue virus[23] and Chikungunya virus.[24] A previous in vitro study showed that murine coronaviruses rely on MMPs for cell fusion and viral replication.[25] The pathologic features of COVID-19 closely resemble those of other SARS-CoV infections, where MMPs play an important role in disease pathogenesis.[26] Therefore, doxycycline may potentially be effective for the treatment of COVID-19 infection.

Ivermectin and doxycycline were co-administered in the treatment group because their synergistic action may increase the likelihood of efficacy in the treatment of COVID-19. We did not observe known drug–drug interactions between ivermectin and doxycycline;[27] therefore, no drug dosage modification was required in the present study.

The effective dose of ivermectin required to reach IC_{50} at a pulmonary level is considerably higher than that used in this study.[20] However, evaluation at higher doses requires detailed safety analysis, which was not within the scope of the present analysis. Therefore, approved dosing regimens of ivermectin and doxycycline were used in this study.

Most patients in our study received the study intervention within 3 to 5 days of symptom onset. No similar study of ivermectin for COVID-19 treatment could be identified in the literature. In the previous remdesivir trial,[19] treatment benefits were prolonged in severe cases (11 vs. 15 days). In the present study, most patients recovered in both the treatment and placebo groups. However, in the treatment group, a higher proportion of patients responded within 7 days than in the placebo group. The proportions of patients whose level of disease severity worsened and who showed virus persistence beyond 14 days were also lower in the active drug group than in the placebo group. Therefore, the ivermectin and doxycycline combination might play a role in early recovery and viral clearance, thus reducing the need for hospitalization and the quarantine period of patients.

This study was performed in patients aged >18 years, and most of the included patients were relatively young (aged <40 years). Therefore, the treatment response we observed might not be generalizable to all age groups. Moreover, the subgroup analysis showed heterogeneous responses; female sex, older patients, and patients with high disease severity showed no significant responses. Early intervention also influenced the response to treatment. Early in the course of the disease, viral replication is particularly high, and in patients with severe disease, viral pathogenicity plays a less dominant role in disease pathogenesis.[28] This observation explains the heterogeneity of the response in relation to the interval of drug administration and disease severity. Pharmacokinetic analysis of ivermectin[29,30] showed that the drug concentration varies according to species, age, sex, and physiological conditions. The effect size of the sample might also play a role in the heterogeneous findings we observed.

FOI 26-3132 - Document 5

Our study findings are suggestive of the efficacy of this combination treatment against COVID-19 infection. However, we speculate that earlier drug administration and a higher dose of ivermectin than that used in the present study might be more beneficial, as a higher dose will help reach the required IC_{50}[20] at the pulmonary level.

We used approved dosing regimens for both drugs and noted very few adverse reactions. Furthermore, most reactions were consistent with those associated with doxycycline,[31] which supports our assumption that the combination treatment was safe to use in the study population. The death rate observed (three of 200 patients in the placebo group; 1.5%) was consistent with the COVID-19 mortality rate in Bangladesh (1.4%).[32]

Our study was performed at a single center over a short period. Therefore, our findings need to be carefully interpreted. Furthermore, a priori sample size calculations were not performed, limiting the strength of our findings. We could not test for viral load, and therefore could not directly assess viral clearance. It remains unclear whether the early reductions in viral load we observed should be verified by repeated RT-PCR testing during follow-up. Our testing facilities did not allow us to test patients repeatedly. Finally, we evaluated the combination of doxycycline and ivermectin but did not determine their individual effects. Therefore, further studies are needed to clarify the results observed in the present study.

Conclusions

Adult patients with mild-to-moderate COVID-19 infection treated with ivermectin combined with doxycycline recovered earlier than those receiving placebo, were less likely to progress to a serious disease, and were more likely to test negative for COVID-19 at the end of the treatment period. Although additional research on the effects of ivermectin combined with doxycycline is warranted, the safety and efficacy of this combination are favorable compared with current standard of care.

Acknowledgments

We wish to thank Popular Pharmaceuticals Bangladesh Limited for supplying the study intervention and personal protective equipment. We also thank Mr. Monir Hossain, the triage room assistant, for assistance with triage, Thomas A. Lang of Tom Lang Communication and Training International for his kind review of the manuscript, and Editage (www.editage.com) for English language editing.

Declaration of conflicting interest

The authors declare that there is no conflict of interest. Popular Pharmaceuticals Limited, Bangladesh provided ivermectin, doxycycline, and placebo. The company was not involved in the planning or design of the study and had no role in the collection, analysis, or interpretation of the data.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Author contributions

R. Mahmud and Md. M. Rahman had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. R. Mahmud, Md. M. Rahman, I. Alam, K. Gias Uddin Ahmed, and S.K. Jakaria Been Sayeed conceived the research and designed the study. R. Mahmud, Md. M. Rahman supervised the research. R. Mahmud, Md. M. Rahman, I. Alam, K. Gias Uddin Ahmed, A.K.M. Humayon Kabir, and Mohammad Zaid Hossain provided administrative, technical, or material support. R. Mahmud, S.K. Jakaria Been Sayeed, and M. Abdullah Yusuf performed the statistical analysis. R. Mahmud, Md. M. Rahman, S.K. Jakaria Been Sayeed, and M. Abdullah Yusuf drafted the manuscript.

FOI 26-3132 - Document 5

All authors were responsible for acquisition, analysis, or interpretation of data and critical revision of the manuscript for important intellectual content.

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FOI 26-3132 - Document 5

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Appendix

Appendix 1. Adverse outcomes

Attribute	Total patients (n = 400)	Treatment group (n = 200)	Placebo group (n = 200)	*P*-value
Death	3 (0.75%)	0	3 (1.5%)	0.016
Adverse drug reaction	9 (2.25%)	9 (2.25%)	0	0.010
Non-ulcer dyspepsia	7 (1.9%)	7 (3.8%)	0	
Erosive esophagitis	2 (0.5%)	2 (1.1%)	0	

FOI 26-3132 - Document 5

Appendix 2. Analysis of patients by death.

Characteristic	Dead (n = 3)	Alive (n = 397)	*P*-value	Risk ratio (95% CI)
Treatment group, placebo (n = 200) n (%)	3 (100)	197 (49.6)	0.25	2.0 (1.8–2.2)
Age, mean (SD) years	63.7 (15)	39.4 (13)	0.001	
Sex, male, n (%)	03 (100)	231 (58.4)	0.27	1.7 (1.6–1.9)
Symptoms at presentation, patients, n (%)				
Fever	3 (100)	297 (74.8)	0.58	1.3 (1.3–1.4)
Cough	3 (100)	244 (61.5)	0.29	1.6 (1.5–1.8)
Running nose	0 (0)	36 (9.1)	>0.99	1.1 (1.0–1.1)
Respiratory distress	2 (66.7)	121 (30.5)	0.22	2.2 (0.9–4.9)
Chest pain	0 (0)	25 (6.3)	>0.99	1.1 (1.0–1.1)
Diarrhea	0 (0)	32 (8.1)	>0.99	1.1 (1.0–1.1)
Vomiting	2 (66.7)	18 (4.5)	0.007	14.7 (5.9–36.8)
Anorexia	1 (33.3)	118 (29.7)	>0.99	1.1 (0.22–5.6)
Anosmia	1 (33.3)	153 (38.5)	>0.99	0.87 (0.17–4.3)
Lethargy	0 (0)	106 (26.6)	0.57	1.4 (1.3–1.4)
Body ache	1 (33.3)	72 (18.1)	0.46	1.8 (0.37–9.2)

SD, standard deviation; CI, confidence interval.

FDA-2023-D-0922 Document 5

J Antimicrob Chemother 2012; **67**: 1884–1894
doi:10.1093/jac/dks147 Advance Access publication 25 April 2012

Journal of Antimicrobial Chemotherapy

Ivermectin is a potent inhibitor of flavivirus replication specifically targeting NS3 helicase activity: new prospects for an old drug

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Received 31 January 2012; returned 6 March 2012; revised 21 March 2012; accepted 26 March 2012

Objectives: Infection with yellow fever virus (YFV), the prototypic mosquito-borne flavivirus, causes severe febrile disease with haemorrhage, multi-organ failure and a high mortality. Moreover, in recent years the *Flavivirus* genus has gained further attention due to re-emergence and increasing incidence of West Nile, dengue and Japanese encephalitis viruses. Potent and safe antivirals are urgently needed.

Methods: Starting from the crystal structure of the NS3 helicase from Kunjin virus (an Australian variant of West Nile virus), we identified a novel, unexploited protein site that might be involved in the helicase catalytic cycle and could thus in principle be targeted for enzyme inhibition. *In silico* docking of a library of small molecules allowed us to identify a few selected compounds with high predicted affinity for the new site. Their activity against helicases from several flaviviruses was confirmed in *in vitro* helicase/enzymatic assays. The effect on the *in vitro* replication of flaviviruses was then evaluated.

Results: Ivermectin, a broadly used anti-helminthic drug, proved to be a highly potent inhibitor of YFV replication (EC_{50} values in the sub-nanomolar range). Moreover, ivermectin inhibited, although less efficiently, the replication of several other flaviviruses, i.e. dengue fever, Japanese encephalitis and tick-borne encephalitis viruses. Ivermectin exerts its effect at a timepoint that coincides with the onset of intracellular viral RNA synthesis, as expected for a molecule that specifically targets the viral helicase.

Conclusions: The well-tolerated drug ivermectin may hold great potential for treatment of YFV infections. Furthermore, structure-based optimization may result in analogues exerting potent activity against flaviviruses other than YFV.

Keywords: antiviral drug discovery, flavivirus helicase inhibition, new use of existing drug, *in silico* docking, structure-based drug design

Introduction

The genus *Flavivirus* comprises small single-stranded RNA viruses within the *Flaviviridae* family. The flavivirus group includes several pathogens of global medical importance, namely (i) haemorrhagic fever viruses such as yellow fever virus (YFV) and dengue viruses (DENV), and (ii) encephalitic viruses such as West Nile virus (WNV), Japanese encephalitis (JEV) and tick-borne encephalitis viruses (TBEV).[1] Infections by either of the last two neurotropic viruses may result in life-threatening aseptic encephalitis, with a high risk of life-long debilitating neurological sequelae.

YFV is the type member of the emerging and re-emerging vector-borne flaviviruses. Infections with YFV cause a severe febrile disease with haemorrhage, multi-organ failure and shock, and an exceedingly high mortality (up to 50% of

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cases).[2,3] YFV is a zoonotic agent that, even with the availability of a safe and efficient vaccine, continues to be reintroduced from sylvatic animal reservoirs into the human population, causing outbreaks in endemic regions of South America and Africa on a regular, yet poorly predictable, basis with an estimated annual number of cases of >200000.[4,5] Moreover, recent increases in the density and distribution of the urban mosquito vector, *Aedes aegypti*, as well as the rise in global air travel, increase the risk of introduction and spread of YFV to North and Central America, the Caribbean and Asia.[2] A major gap in our knowledge about YFV is how to manage and treat patients. Treatment of YFV by supportive care is essentially ineffective, and even improvements in intensive care have not changed the lethality.[3] Likewise, the four DENV serotypes have considerably expanded their geographic distribution in recent years. With billions of people at risk, more than 50 million cases, and ~12500–25000 deaths annually, DENV is considered an emerging pathogen in a growing number of countries.[6] In particular, the presence of four DENV serotypes has complicated the design of vaccines because incomplete protection against one serotype may influence the disease outcome once infection is established by a different serotype, through a process referred to as antibody-mediated disease enhancement.[7]

Annually there are ~30000–50000 cases of JEV reported in Asia. Case-fatality rates range from 0.3% to 60%. In Russia and Europe, TBEV causes ~10000–12000 human cases, which may present with severe clinical presentations and a significant rate (10%–20%) of long-lasting neurological sequelae in survivors. WNV was detected for the first time in the Americas in 1999; the virus is now endemic on this continent.[5,8] There is thus an urgent need for antiviral drugs to treat life-threatening infections with flaviviruses.

During the flavivirus replication cycle, the viral genome is transcribed into a negative-strand RNA, which in turn is used as a template for the synthesis of new viral genomic RNA. To maintain viral replication the nascent transcripts must be unwound from their complementary template RNAs by an ATP-dependent helicase activity. In flaviviruses this activity is provided by the C-terminal domain of non-structural (NS) protein 3 (NS3 helicase domain).[9–11] Since all flaviviral NS proteins are indispensable for viral replication, any of them may be considered a promising target for selective inhibitors of viral replication for therapeutic intervention.[12] Moreover, in addition to the commonly targeted viral polymerase and protease functions, the feasibility of interfering with viral replication by particularly inhibiting RNA helicase activity has been a successful proof of concept for hepatitis C virus (HCV)[13] and picornaviruses.[14] For flaviviruses, a number of nucleoside 5′ triphosphates (TPs), such as ribavirin-TP[15–17] (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-triphosphate), IDA-TP (4,6-diamino-8-imino-8*H*-1-β-D-ribofuranosylimidazo [4,5-e] [1,3]diazepine-5′-triphosphate) and ITA-TP[18] (5,8-dioxo-5,6,7,8-tetrahydro-4*H*-1-β-D-ribofuranosylimidazo[4,5-e][1,2,4]triazepine-5′-triphosphate) have been reported as weak inhibitors of the ATPase activity of helicases from HCV, JEV, DENV and WNV.[16,18,19] However, since ATP is a key nucleotide of host cell metabolism, ATP-mimetic molecules may result in adverse effects on the host cell. A more innovative helicase inhibitor development strategy (as demonstrated to some extent for HCV[20]) might be directed specifically against the RNA binding and unwinding mechanisms mediated by NS3, which have recently been unravelled in fine molecular detail.[11,21]

Based on the above considerations, we performed an *in silico* docking search targeting a selected region of the ssRNA access site in the crystal structure of the NS3 helicase domain[21,22] of Kunjin virus (an Australian variant of WNV, to which we refer as WNV[23]), using a library of mostly commercial small molecules. We identified the widely used anti-helminthic drug ivermectin as a molecule that not only displayed a high predicted binding affinity towards the modelled NS3 ssRNA binding pocket, but also inhibited the NS3 helicase activity of several flaviviruses *in vitro* at sub-micromolar concentrations. Most importantly, ivermectin proved to be a selective inhibitor of the replication of several flaviviruses in cell culture, such as JEV, TBEV and DENV (sub-micromolar EC_{50} values), and a highly potent inhibitor of YFV replication (sub-nanomolar EC_{50} values). Considering that this well tolerated drug has been licensed for >20 years for the treatment of parasitic infections in man, our results provide the prospect of the first specific anti-flavivirus therapy by the off-label use of ivermectin (patent application EP2010/065880).

Materials and methods

Chemical database for virtual screening and reagents

The virtual Library of Pharmacologically Active Compounds (LOPAC) used for the docking analysis was accessed from Sigma-Aldrich, and included 1280 commercially available compounds (www.sigmaaldrich.com). The compounds tested *in vitro*, paromomycin sulphate, ouabain and ivermectin, were obtained from Sigma-Aldrich; ribavirin (Virazole) was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). The compounds were dissolved at 20 mM in DMSO and stored at −20°C. The compounds were used as provided, without further purification.

In silico search for NS3 helicase inhibitors

The AutoDock4 software package[24] was used for a docking search using compounds from the LOPAC library, and Python Molecule Viewer 1.4.5 (MGL-tools package, http://mgltools.scripps.edu/) for analysis of the data. The atomic coordinates from the crystal structure of the WNV helicase domain, solved in our laboratory, were chosen as the docking model (PDB 2QEQ[21]). After the addition of hydrogen atoms (PMV, MGL Tools package, http://mgltools.scripps.edu/), Kollman charges[25] were added to the model. A discrete grid with dimensions 23×19×15 Å^3 (AutoGrid4; step size 0.375 Å, 62×50×40=124000 points[26]) was then centred on the putative ssRNA access site between helices α2 in subdomain II and α9 in subdomain III. Twenty genetic algorithm searches were run using AutoDock4 for each compound (provided with Gasteiger charges[27]) in the LOPAC library (with 150 individuals in the population and 27000 generations[24]). The docking search produced a ranked list of compounds with predicted free energy of binding (ΔG) ranging between +9.0 and −11.5 kcal/mol. The best three molecules [paromomycin sulphate, ouabain and ivermectin (form B1a from Sigma-Aldrich, modelled with 11 rotatable bonds)], displaying ΔG values between −11.5 and −9.5 kcal/mol, were selected to be tested in *in vitro* activity assays.

Expression and purification of NS3 and NS5 domains

The WNV, DENV serotype 2 and YFV helicase domains were expressed and purified as previously described.[28,29] DENV RNA-dependent RNA polymerase (RdRp), DENV full-length NS5, WNV RdRp and WNV full-length NS5 were produced in an *Escherichia coli* Rosetta (DE3) pRos expression

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system and purified through a Ni^{2+} column and gel filtration (Hi Load 16/60 Superdex 200, GE Healthcare) as described for the WNV helicase domain.[28,29]

Helicase inhibition assays using radioactive and fluorescent (FRET-based) labels

The helicase activity was assayed using radiolabelled dsRNA substrate in the presence of Mg^{2+} and ATP. The dsRNA substrate was prepared as described previously.[30] Briefly, primer 1 (5′-CACCUCUCUAGAGUCGACCUGCAGGCAUCG-3′) was labelled with [γ-^{32}P]ATP at its 5′ end using T4 polynucleotide kinase and annealed with the complementary primer 2 (5′-CGACUCUAGAGAGGUG-3′). WNV NS3 helicase (200 nM, see below) was preincubated with various concentrations of ivermectin (between 5 and 400 nM) in 50 mM Tris/HCl pH 8.0, 5 mM dithiothreitol, 10 mM KCl, 20 U/mL RiboLock Ribonuclease Inhibitor (Fermentas), 5 mM $MnCl_2$ and 5 mM $MgCl_2$. The reactions were initiated by adding the proteins to the reaction mixture containing 6 fmol of dsRNA, and were quenched after 30 min at 37°C by addition of 6 μL of loading dye (50 mM EDTA, 0.5% SDS, 50% glycerol and 0.1% bromophenol blue). The assay mixtures were resolved by electrophoresis through 17% polyacrylamide gels that were dried and analysed by phosphoimaging (Typhoon; GE Healthcare).

Fluorescence helicase assays were performed as described by Boguszewska-Chachulska *et al.*[31] Briefly, the substrate for the fluorescent helicase test was prepared by annealing, at a 1:1.2 molar ratio, a Cy3-labelled 30-mer (5′-CACCUCUCUAGAGUCGACCUGCAGGCAUCG-3) to a Black Hole Quencher (BHQ)-labelled 16-mer (5′-CGACUCUAGAGAGGUG-3′), by brief heating to 90°C then slow cooling to room temperature. Standard helicase assays were performed in 50 mM Tris/HCl pH 7.5, 5 mM dithiothreitol, 10 mM KCl, 20 U/mL RiboLock Ribonuclease Inhibitor (Fermentas), 5 mM $MnCl_2$ and 5 mM $MgCl_2$ (buffer 1), 20 nM substrate, 1 mM ATP and 250 nM capture strand (5′-CGACUCUAGAGAGGUG-3′) in a reaction volume of 200 μL. The YFV, YFV-double mutant, DENV, DENV-double mutant and WNV, WNV-double mutant helicase domains (200 nM) were pre-incubated with various concentrations of ivermectin. The unwinding reaction was started by addition of helicases or ATP. The fluorescent signal increase was measured using a Fluorescence Reader (Infinite 200; Tecan). Relative fluorescence was calculated by subtracting the mean fluorescence of the blank (assay without protein) from all samples, and the values obtained were converted to percentage of activity.

The HCV helicase activity assay in the absence and presence of eight concentrations of ivermectin (0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM) was performed as described previously.[32] In brief, the reaction mixture [25 mM MOPS pH 6.5, 1.25 mM MgCl2, 5 nM Cy5-4-methylbenzhydrylamine hydrochloride (MBHA) substrate (DNA), 12.5 nM HCV NS3 helicase, 0.05 mM dithiothreitol, 0.01% Tween 20, 5 μg/mL BSA and 1 mM ATP] and ivermectin were added to a white half-volume 96-well microplate using EPmotion. Fluorescence was monitored using the VarioSkan. The reaction was incubated for 2 min before VarioSkan injection of ATP.

Helicase kinetics and inhibition by ivermectin

RNA unwinding was measured under the same experimental conditions as those described above, with dsRNA concentrations ranging from 0 to 100 nM, in the absence or presence of ivermectin at various concentrations. After blank subtraction (the same mixture in the absence of the enzyme), the curve was fitted linearly to get the rate of product formation. Different rates measured at varying substrate concentrations were plotted according to the Lineweaver–Burk equation, with amounts of dsRNA (substrate) ranging from 0 to 100 nM, to get V_{MAX} and K_m. The same experiment performed at increasing inhibitor concentrations yielded parallel lines in the double reciprocal plot, indicating that ivermectin behaves as an uncompetitive inhibitor. To estimate the inhibition constant (K_i) we used the Lineweaver–Burk equation for an uncompetitive inhibitor extrapolated for infinite substrate concentration:

$$\frac{1}{v} = \frac{K_m}{[S]v_{MAX}} + \frac{1}{v_{MAX}}\left(1 + \frac{[I]}{K_i}\right) \underset{[S]\to\infty}{\rightarrow} \frac{1}{v_{MAX}}\left(1 + \frac{[I]}{K_i}\right)$$

Double mutation of YFV, DENV and WNV helicase domain

The double YFV (T413I and D414E), DENV helicase mutant (T408I and D409E) and WNV double mutant (T409I and D410E) were produced using the primer design software provided by the Agilent Technologies web site (www.agilent.com/genomics/qcpd). The primers for YFV were as follows: sense (5′-GGGACTTTGTCGTCACAATAGAGATATCTGAGATGGGAGCA-3′); antisense (5′-TGCTCCCATCTCAGATATCTCTATTGTGACGACAAAGTCCC-3′).

The primers for DENV were as follows: sense (5′-GGGACTTCGTGGTCACAATTGAGATTTCAGAAATGGGTGCC-3′); antisense (5′-GGCACCCATTTCTGAAATCTCAATTGTGACCACGAAGTCCC-3′).

The primers for WNV were as follows: sense (5′-GGGACTTTGTCGTCACAATAGAGATATCTGAGATGGGAGCA-3′); antisense (5′-TGCTCCCATCTCAGATATCTCTATTGTGACGACAAAGTCCC-3′).

The proteins were expressed and purified as previously described.[28,29]

Biophysical characterization of the NS3–ivermectin interaction

Thermofluorimetric (thermal shift) assays for the evaluation of the YFV, WNV and DENV helicase domain melting temperatures (T_m) in the absence/presence of ivermectin were conducted in a MiniOpticon Real Time PCR Detection System (Bio-Rad), using the fluorescent dye Sypro orange. Solutions of 2.5 μL of the helicase domain (final protein concentrations ranged between 0.5 and 5 mg/mL) were diluted in 19 μL of buffer 1 and mixed with 3.5 μL of Sypro orange (Sigma) diluted 60× and 0.5 μL of 2 mM ivermectin. In control samples the inhibitor was replaced by DMSO. The sample plates were heated from 25 to 95°C with a heating rate of 0.2°C/min. Fluorescence intensities were measured within excitation and emission ranges of 470–505 and 540–700 nm, respectively. The experiments were repeated in the presence of 0.5 μL of 50 μM dsRNA, prepared by annealing at a 1:1 molar ratio of primer 1 (5′-CACCUCUCUAGAGUCGACCUGCAGGCAUCG-3′) and complementary primer 2 (5′-CGACUCUAGAGAGGUG-3′).

NS3 ATPase assay

The luciferase/luciferin-based ATP Detection Kit (Sigma–Aldrich) was prepared according to the manufacturer's instructions. Twenty microlitres of luciferase/luciferin was added to 185.5 μL of reaction buffer and the reactions were initiated by adding 10 μg of YFV, DENV or WNV helicase and 2.5 μL of ATP (100 mM). In parallel experiments, the YFV, DENV or WNV helicase domain was incubated with ivermectin to a final concentration of 1 μM at 30°C for 10 min. In control samples, the DENV or WNV helicase domain was omitted from the reaction mixture. All luminescence measurements were performed with a Cary Eclipse Fluorescence Spectrophotometer (Varian) at 25°C. Luminescence was measured continuously for 30 min (1 s reading time).

NS5 RdRp activity assay

In vitro RNA synthesis assays were performed using poly(C) (MP Biomedicals) as template annealed with oligo$(G)_{12}$ as primer (62.5 nM final concentration) and GTP (100 mM final concentration) as substrate, in a 200 μL reaction mixture containing buffer 1, PicoGreen Quantitation Reagent (Molecular Probes) and 1 μg of DENV RdRp, DENV full-length

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NS5, WNV RdRp or WNV full-length NS5. Ivermectin was added to a final concentration of 1 μM. Standard assays were performed after enzyme/drug preincubation for 10 min at room temperature. Reactions were started by the addition of GTP and incubated for 15–20 min at 25°C following the fluorescence of the samples in a fluorescence reader (Cary Eclipse Fluorescence Spectrophotometer). Relative fluorescence was calculated by subtracting the mean fluorescence of the blank (assay without protein) from all samples.

Viruses and cells

YFV 17D vaccine strain (Stamaril) [Aventis Pasteur (MSD, Brussels, Belgium)] and DENV serotype 2 New Guinea were passaged once in Vero-B cells (ATCC CCL-81) to prepare a working virus stock and stored at −80°C until further use; JEV strain SA-14 (GenBank accession number U14163), TBEV strain Oshima 5-10 (GenBank accession number AB062063) and WNV strain NY99 (GenBank accession number NC_009942) were passaged once in Vero E6 cells (ATCC C1008) to prepare a working virus stock and stored at −80°C until further use.

Cytotoxic and cytostatic assays

Potential cytotoxic effects of the compounds were evaluated in uninfected quiescent Vero-B and Vero E6 cells. The cells were seeded at 5×10^4 cells/well in a 96-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in the presence of 2-fold serial dilutions and incubated for 4 days. The culture medium was discarded and 100 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium/phenazinemethosulphate (MTS/PMS; Promega, Leiden, the Netherlands) in PBS was added to each well. Following a 2 h incubation at 37°C, the optical density was determined at 498 nm. The cytotoxic activity was calculated using the following formula: percentage host cell metabolism $=100\times(OD_{Compound}/OD_{CC})$, where $OD_{Compound}$ and OD_{CC} are the optical density at 498 nm of the uninfected cell cultures treated with the compound and of the uninfected, untreated cell cultures, respectively. The 50% cytotoxic concentration (i.e. the concentration that reduces the total cell number by 50%; CC_{50}) was calculated by logarithmic interpolation.

Antiviral cell-based assays

YFV-17D cytopathic effect (CPE)-based assay

Vero-B cells were seeded at a density of 2×10^4 cells/well (96-well plate, Falcon) in 100 μL of assay medium and allowed to adhere overnight. Subsequently, a compound dilution series was prepared in the assay medium on top of the cells, after which 100 μL of assay medium containing 100 $CCID_{50}$ (50% cell culture infectious dose) of virus was added. Plates were incubated for 6 days at 37°C (95%–99% relative humidity and 5% CO_2). The cells were fixed with 70% ethanol and stained with 1% methylene blue. Pictures were taken at ×100 magnification. Ribavirin was included as a reference compound. The potential cytotoxic effect of the compounds was evaluated in uninfected cells in a parallel assay with the same experimental setup.

Virus yield reduction assays

DENV serotype 2 and YFV-17D Vero-B cells (5×10^4) were seeded in 96-well plates. One day later, culture medium was replaced with 100 μL of assay medium containing a 2× serial dilution of the compounds and 100 μL of virus inoculums (50 $CCID_{50}$/μL). Following a 2 h incubation period the cell monolayer was washed three times with assay medium to remove non-adsorbed virus and cultures were further incubated for 4 days in the presence of the inhibitor. Supernatant was harvested and viral RNA load was determined by quantitative real-time PCR (qRT-PCR) as described previously.[33]

TBEV strain Oshima 5-10, JEV strain SA-14 and WNV strain NY99. Antiviral activity was assessed in Vero E6 cells grown in 24-well tissue culture plates. Viruses were used at a dilution of moi <1 (i.e. between 0.1 and 1). The compounds were used at a final concentration ranging between 0 and 5 μM. Pre-incubation in the presence of the antiviral was performed during the 90 min virus adsorption step. The cells were then washed once and incubated for 3 days in the presence of the antiviral compounds. All conditions were assayed in duplicate. Three days (72 h) post-infection, each corresponding cell culture supernatant was removed, clarified by centrifugation and stored at −80°C before analysis using a viral titre reduction assay.

The viral titre reduction assay was performed using BHK21 cells seeded in 96-well plates. When the cells reach 80% confluence, they were infected with 150 μL of 10-fold serial dilutions of the virus (derived from the Vero cell clarified supernatants) for 4 days before microscopic examination and positive cytopathic effect (CPE) well counting. For each supernatant sample, the infectivity titre was expressed as $TCID_{50}$/mL using the Karber formula. For each virus, titres of viral supernatants were then compared and represented as the percentage of the positive control (viral titre from infected cell supernatant sample without antiviral compound) for the calculation of EC_{50} values. For WNV, the viral RNA load was also determined by real-time qRT-PCR as described previously.[33]

Time-of-drug-addition study

One day prior to infection, Vero-B cells were seeded in a 24-well Falcon plate (2×10^5 cells/well). Ivermectin (50 nM) was added at 0, 2, 4, 6, 8 and 24 h after YFV-17D infection (50 $CCID_{50}$). In parallel experiments, ribavirin (400 μM) was used as a reference compound. Cells from each well were collected at 24 h after virus infection. For RNA extraction, at 24 h post-infection, cells were washed with PBS and lysed with lysis buffer (buffer RLT, RNeasy Mini Kit; Qiagen) to obtain cytoplasmic extracts. Cytoplasmic RNA was then extracted according to the manufacturer's instructions and analysed for the presence of viral RNA by RT-qPCR using a standard curve. Intracellular viral RNA production was monitored during one replication cycle in untreated cells. Confluent Vero-B cells in a 24-well plate were infected and incubated at 37°C for 1 h. After washing, 700 μL of assay medium was added and cells were harvested every 2 h. RNA replication was monitored by measuring intracellular RNA by RT-qPCR following reverse transcription. Time-of-addition studies using full-length dengue reporter virus expressing *Renilla* luciferase were performed as described previously.[33]

Results

Identification of ivermectin as putative flavivirus NS3 inhibitor by in silico docking

To identify potential NS3 helicase inhibitors, we chose to target the ssRNA access site of the enzyme in an *in silico* docking library search. Since the structure of DENV helicase in complex with RNA[11] was not available when we initiated this work, we modelled the flaviviral NS3 helicase/RNA interaction by superposition of the structure of WNV NS3 helicase (solved in our laboratory, PDB 2QEQ[21]) and the DNA-bound bacterial helicase PcrA (PDB 3PJR[34]). This model was used to locate the putative ssRNA access site within the WNV NS3 helicase domain (Figure 1a). This region is located between helices α2 in subdomain II and α9 in subdomain III (forming an 'α-helical gate'); this model-based identification was later confirmed by the

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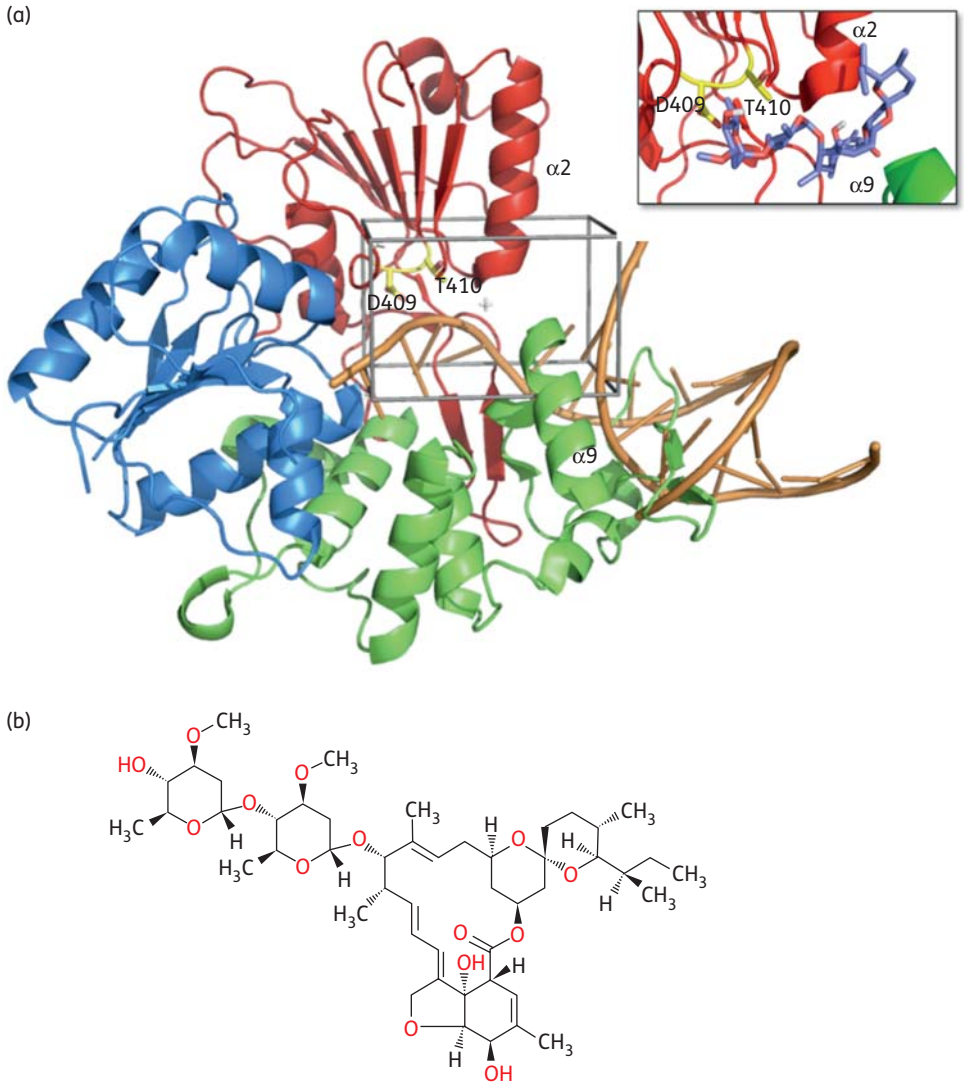


Figure 1. *In silico* docking of flavivirus helicase. (a) Model of the helicase/RNA interaction for WNV helicase domain. The crystal structure of free WNV helicase (PDB 2QEQ) was used to identify a potential ssRNA access site to the enzyme active site by superposition with the DNA-bound bacterial helicase PcrA (PDB 3PJR). The respective region is located between helices α2 in subdomain II (red) and α9 in subdomain III (green) of the WNV helicase, forming an α-helical gate for the entering RNA substrate (orange worm/sticks). This putative helicase ssRNA access site (enclosed in black) was then chosen as the target site for the *in silico* ligand search. The amino acids selected for mutation (Asp409 and Thr410) are shown in yellow. The close-up box shows a potential docking conformation of ivermectin in the ssRNA access site. The location of the molecule's macro ring is well established, having the two sugar rings of the compound located inside the protein. The region occupied by the inhibitor is conserved in all binding modes of the different docked conformations. (b) Chemical structure of ivermectin (B1a form; Sigma-Aldrich). Figures created using PyMol (http://www.pymol.org). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

crystal structure of DENV in complex with ssRNA.[11] The selected target site was then explored *in silico* by docking low molecular weight compounds from the LOPAC library (see the Materials and methods section). This docking search (AutoDock4[24]) resulted in the identification of three molecules with calculated binding free energy (ΔG) for WNV NS3 helicase ranging from −11.5 to −9.5 kcal/mol; the compounds were ivermectin (a macrocyclic lactone antibiotic with broad-spectrum antiparasitic activity, Figure 1b), ouabain (a cardiac glycoside) and paromomycin sulphate (an aminoglycoside antibiotic used to treat, among others, cutaneous leishmaniasis). These molecules were selected for further biochemical evaluation in *in vitro* helicase activity assays.

Specific inhibition of flaviviral NS3 helicase unwinding activity

The inhibitory effects of the three selected molecules (ivermectin, ouabain and paromomycin sulphate) on flavivirus helicase activity were evaluated against the helicase domain of WNV. Helicase inhibition activity was assayed using a radiolabelled

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RNA substrate in the presence of Mg^{2+} and ATP.[30] For this purpose, the NS3 helicase domain was pre-incubated with various concentrations of the inhibitors. Ouabain and ivermectin inhibited the dsRNA unwinding activity of WNV helicase with an IC_{50} between 200 and 400 nM (not shown). Paromomycin sulphate showed strong inhibitory activity at all concentrations tested (as low as 0.5 nM), likely due, however, to its non-specific RNA-binding capacity, reported previously.[35]

Using a complementary FRET-based helicase assay (see the Materials and methods section), we demonstrated that the specific inhibitory activity of ivermectin extends further to the NS3 helicase of the YFV helicase with an IC_{50} of 122 ± 10 nM (Figure S1a, available as Supplementary data at *JAC* Online) and DENV with an IC_{50} of 500 ± 70 nM (Figure S1b, available as Supplementary data at *JAC* Online). Using this assay, the activity of ivermectin against the WNV helicase was confirmed, with an IC_{50} of 350 ± 40 nM (Figure S1c, available as Supplementary data at *JAC* Online).

To exclude any effect of the inhibitor on the ATP binding site, the influence of ivermectin on the ATPase activity of the YFV, WNV and DENV helicase domains was assessed. Ivermectin at a concentration of 1 μM did not affect the ATPase activity of the YFV, WNV and DENV helicase domains (Figure S2, available as Supplementary data at *JAC* Online), suggesting that the compound does not restrict the helicase ATP binding site located at a distance of ~25 Å from its predicted binding site in the helicase domain.

On the other hand, the inhibition of helicase activity does not result from ligand-induced protein destabilization/aggregation/denaturation, as demonstrated by thermofluorometric assays. In fact, YFV, DENV and WNV helicases displayed essentially the same T_m in the presence and absence of ivermectin, with or without dsRNA (not shown).

To rule out any off-target effect of ivermectin on the flaviviral NS5 RdRp, polymerase activity assays were performed using both the RdRp domains and the NS5 full-length proteins from DENV and WNV. The polymerase activity of any of the four proteins was not affected by the presence of 1 μM ivermectin (Figure S3, available as Supplementary data at *JAC* Online).

Finally, to verify the specificity of ivermectin for the flavivirus helicases, we performed activity assays on HCV helicase. The helicase of HCV, which belongs to the family *Flaviviridae*, is closely related to flavivirus helicases.[30] Tests using HCV helicase in the presence of ivermectin (up to 100 μM) did not show any inhibition of the dsRNA unwinding activity of the enzyme, indicating that ivermectin inhibition is exclusive for flavivirus helicases (not shown).

YFV, DENV and WNV helicase double mutants

In order to characterize extensively the part of the protein involved in ivermectin binding, we analysed, by *in silico* docking, all the possible helicase/ivermectin interactions in the ssRNA access site. Using all the known coordinates of the different flaviviral helicases, we produced a number of virtual conformations of the ligand in the chosen site using the program AutoDock4 (not shown). This virtual search pointed to possible mutations perturbing the predicted binding mode of the inhibitor to the selected site of the protein. In particular, we identified Thr408 and Asp409 (numeration as in DENV helicase PDB 2JLQ) as high-frequency interactors in the different conformations of ivermectin produced by virtual docking. This analysis suggested that the substitution of the two amino acids with others having slightly bulkier side chains could hamper ligand binding. Since the two amino acids are conserved among flaviviral helicases, in order to preserve activity we chose to make two minor mutations: Thr408Ile and Asp409Glu in the DENV helicase domain, Thr409Ile and Asp410Glu in the WNV helicase domain (Figure 1a) and Thr413Ile and Asp414Glu in the YFV helicase domain.

As expected, the mutated YFV, DENV and WNV helicases maintained good dsRNA unwinding activity. In contrast, the mutant helicases were not inhibited by ivermectin (up to 5 μM), indicating that binding of the inhibitor was strongly hampered by the inserted mutations (Figure S1a–c). This indicates that the two amino acids (or one of the two) selected for mutation strongly interact with the inhibitor and consequently that ivermectin actually interacts with the ssRNA access site as predicted by our structural analysis. Notably, both residues are conserved among all the flaviviruses and belong to the conserved motif V in helicase superfamily II.

Helicase kinetics and inhibition

In order to investigate the mechanism of helicase inhibition exerted by ivermectin we performed kinetic assays. It is possible to describe the helicase kinetics by a simple Michaelis–Menten model when neglecting the inhibitory effect due to ADP production during the first 10 min of reaction. The mechanism of inhibition proved to be uncompetitive for all three viral enzymes, with the inhibition constant being 19 ± 0.2, 354 ± 23 and 175 ± 25 nM for YFV, DENV and WNV, respectively (Figure 2a–c), indicating that ivermectin is able to bind effectively to the protein only when RNA is present (enzyme–substrate complex). The inhibition constants calculated by kinetic analysis are reported in Table 1.

Inhibition of in vitro viral replication

The potential inhibitory effects of the identified molecules on *in vitro* replication of different flaviviruses (YFV, DENV and WNV) were evaluated. Among the three compounds, only ivermectin inhibited the replication of the selected viruses. In particular, highly potent inhibition of YFV replication was observed. In a CPE reduction assay in Vero-B cell culture, the EC_{50} for inhibition of YFV replication was between 3.1 and 6.3 nM (Figure 3a), whereas in virus yield reduction assays the EC_{50} for inhibition of viral progeny formation was in the low sub-nanomolar range (~0.5 nM, Figure 3b and Table 1). Ivermectin proved less active against DENV in the CPE reduction assay (EC_{50} >1 μM, not shown), although inhibition in virus yield reduction assays was observed (EC_{50} 0.7 μM, quantified by qRT–PCR; Table 1). Ivermectin inhibited the production of infectious WNV, although with an EC_{50} of 4 μM (Table 1). Interestingly, the antiviral activity of ivermectin appears to be selective against flaviviruses, as demonstrated by its capability to inhibit the production of infectious TBEV and JEV viruses with EC_{50} ~0.2 and 0.3 μM (CC_{50} of ~10 μM, not shown), respectively, and its failure to inhibit bovine viral diarrhoea virus (BVDV) and HCV (which belong to the related genera pestivirus and hepacivirus, respectively, within the *Flaviviridae* family), but also Coxsackievirus B2

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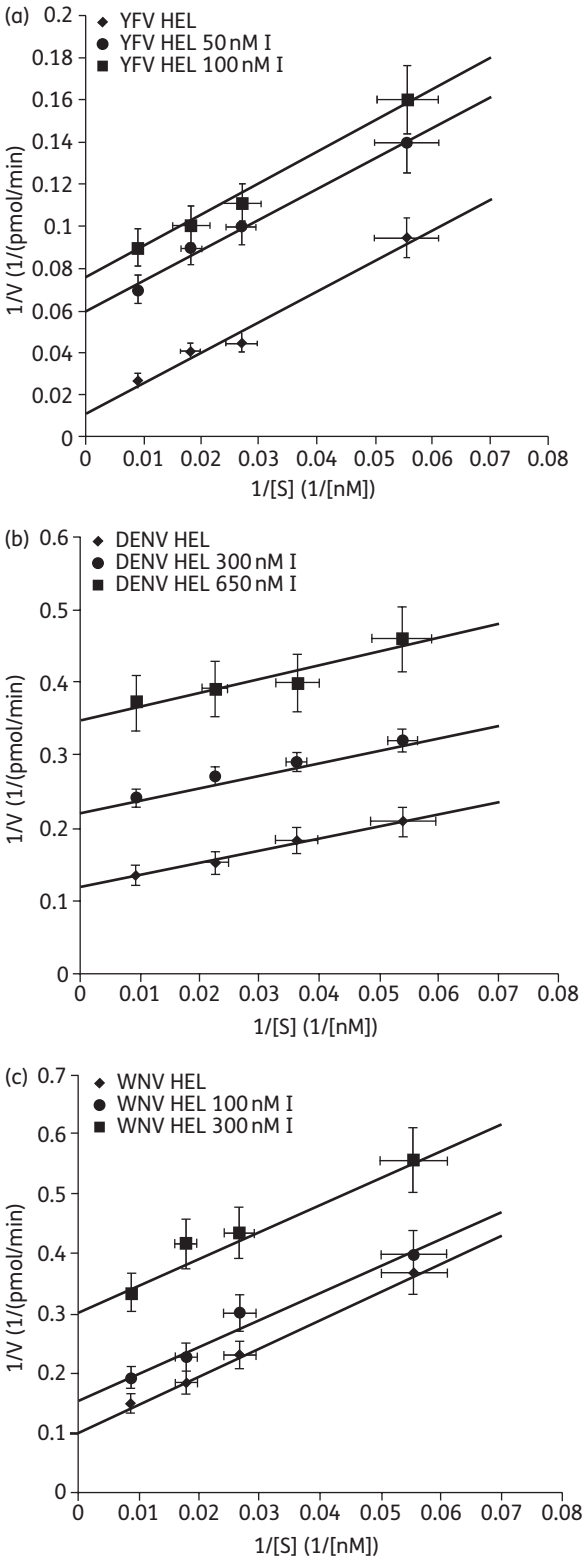


Figure 2. Kinetic studies of the mechanism of helicase inhibition by ivermectin. Double-reciprocal plot of the rate of dsRNA unwinding versus substrate concentration (dsRNA) during the inhibition of (a) YFV helicase, (b) DENV helicase and (c) WNV helicase by an increasing amount of ivermectin (I). HEL, helicase.

(CVB-2) and poliovirus 1 (Sb-1) (*Picornaviridae* family), herpes simplex virus 1 (HSV-1) and vaccinia virus (VV) (*Herpesviridae* and *Poxviridae* family, respectively), vesicular stomatitis virus (VSV) and respiratory syncytial virus (RSV) (*Rhabdoviridae* and *Paramyxoviridae* family, respectively) and Reovirus-1 (Reo-1) (*Reoviridae* family) (data not shown).

Time-of-drug-addition assays

Time-of-drug-addition assays (0–24 h) were performed to examine at which stage in the viral replication cycle (from virus binding to release) ivermectin exerts its antiviral activity on YFV replication. In the virus growth curve without inhibitor, onset of intracellular viral RNA synthesis was at 14 h post-infection (Figure 4a). Ivermectin and the reference molecule ribavirin were highly effective in inhibiting YFV replication when added during the first 14 h after virus infection, but when added at a later stage, i.e. after onset of intracellular viral RNA synthesis (as assessed in the untreated cultures) the molecule lost its antiviral effect (Figure 4b). Comparable results were obtained in the DENV serotype 2 time-of-addition study, performed using the full-length dengue reporter virus expressing *Renilla* luciferase (not shown).

Discussion

Infections with YFV and other emerging and re-emerging pathogenic flaviviruses (such as DENV, WNV and JEV) pose a serious global public health problem.[1] Potent and safe antivirals are urgently needed. Such drugs may be used for the treatment of life-threatening infections with YFV, or encephalitis induced by WNV or JEV infections. Substantial progress has been made in recent years in understanding the biology of replication of flaviviruses, in some cases even in great molecular detail,[12,36] allowing innovative approaches to drug discovery, such as structure-based drug screening and design.[12,37]

We undertook an *in silico* screen for potential inhibitors of the flaviviral NS3 helicase. Starting from the crystal structure of the NS3 helicase domain of WNV,[21] we identified the enzyme's putative ssRNA access site, located between helicase subdomains II and III (α-helical gate; Figure 1a). According to our mechanistic analysis this site might be crucial for the helicase enzymatic function, and thus be a target for inhibition of viral replication. Virtual docking of the LOPAC library to this site identified ivermectin (a broad-spectrum antiparasitic agent), ouabain (a cardiac glycoside) and paromomycin sulphate (an aminoglycoside antibiotic) as potential flavivirus helicase binding compounds. Using *in vitro* enzymatic assays employing recombinant NS3 helicases of flaviviruses from several distantly related serogroups (WNV from the JEV serogroup and YFV and DENV from the YFV serogroup), we could confirm that ivermectin inhibited the dsRNA unwinding activity of different flaviviral helicases (with IC_{50} values in the sub-micromolar range; Figure 2). For ivermectin, the specificity of this inhibition was corroborated by excluding any off-target effects on the helicase-associated ATPase activity and the overall native protein fold (by thermal denaturation analysis).

Extending our *in silico* analysis to all the available structures of flaviviral helicases, we simulated a number of possible conformations of ivermectin inside the ssRNA access site. The results

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Table 1. Effect of ivermectin on the activity of recombinant flavivirus helicases, viral RNA formation in virus-infected cultures and host cell metabolism

Virus	Fluorescent helicase inhibition, IC_{50} (μM)[a]	Helicase kinetics, K_i (μM)[b]	qRT–PCR, EC_{50} (μM)[c]	CC_{50} (μM)[d]
YFV	0.12 ± 0.01	0.019 ± 0.002	0.0005	3.5
DENV	0.50 ± 0.07	0.354 ± 0.023	0.7	3.8
WNV	0.35 ± 0.04	0.175 ± 0.025	4	10

All cell data are presented as averages of three independent experiments and all standard errors were <10%.
[a]Compound concentration required to achieve 50% inhibition of the helicase activity using the fluorescent helicase inhibition assay; helicase domain of YFV = amino acids 184–623, helicase domain of DENV = amino acids 168–618 and helicase domain of WNV = amino acids 180–619.
[b]Inhibition constant calculated from helicase kinetic assays.
[c]Compound concentration required to inhibit viral RNA synthesis by 50% in Vero cells infected with the YFV 17D vaccine strain, the DENV serotype 2 New Guinea C or the WNV strain NY99.
[d]Compound concentration required to reduce the viability of Vero-B cells (for YFV and DENV) and Vero E6 cells (for WNV) by 50%.

allowed the identification of two conserved amino acids (T408 and D409 in DENV) often interacting with the different conformations of ivermectin. This suggests that a quasi-conservative substitution of both amino acids (with slightly bulkier side chains) could hamper ligand binding to the protein. Accordingly, a DENV helicase double mutant (T408I and D409E), a WNV helicase double mutant (T409I and D410E) and a YFV helicase double mutant (T413I and D414E) were generated. The mutated proteins preserved helicase activity but were not inhibited by ivermectin up to a concentration of 5 μM. This provides strong evidence that the two amino acids (or one of the two) strongly interact with the inhibitor, confirming the binding of ivermectin to the ssRNA access site. Notably, both residues are part of the motif V in helicase superfamily II and are conserved among the flaviviruses.

We later showed that ivermectin behaves as an uncompetitive helicase inhibitor, able to bind only to the protein/RNA complex blocking the enzymatic activity. This result is in agreement with the failure to measure binding of ivermectin to the enzyme using micro-calorimetry (not shown) or to obtain crystals of the protein–ligand complex (data not shown). On the basis of the available structures of DENV bound to ssRNA (PDB 2JLU) it is not possible to predict a plausible interaction site or a model of the ternary complex. Reasonably, during activity the helicase/RNA complex changes its structure,[38] allowing ivermectin to interact with the identified amino acids to block dsRNA unwinding.

Most importantly, we demonstrated that ivermectin (and not ouabain or paromomycin sulphate) inhibited the *in vitro* replication of different flaviviruses. Ivermectin proved most potent against YFV, but also inhibited, although less efficiently, the *in vitro* replication of DENV, JEV, WNV and TBEV. The highly potent antiviral effect of ivermectin against YFV (EC_{50} ~0.5 nM) is striking, considering that other recently reported inhibitors of flavivirus replication display activity that is several orders of magnitude lower (NITD008, EC_{50} 2 μM[39] and NITD-618, EC_{50} 1–4 μM,[40] both against DENV; 2′-*C*-methylcytidine, EC_{50} 100 μM[41] and T-705, EC_{50} 330 μM,[42] both against YFV).

We demonstrated in a time-of-drug-addition assay that ivermectin exerts its anti-YFV activity when administered during the first 14 h after virus entry into cells (Figure 4b). In fact, the compound gradually loses its antiviral potency when first added to YFV-infected cultures after onset of intracellular viral RNA synthesis (Figure 4a). Thus, the compound is effective only during that particular phase of the flaviviral replication cycle in which the viral helicase is functionally active.[36]

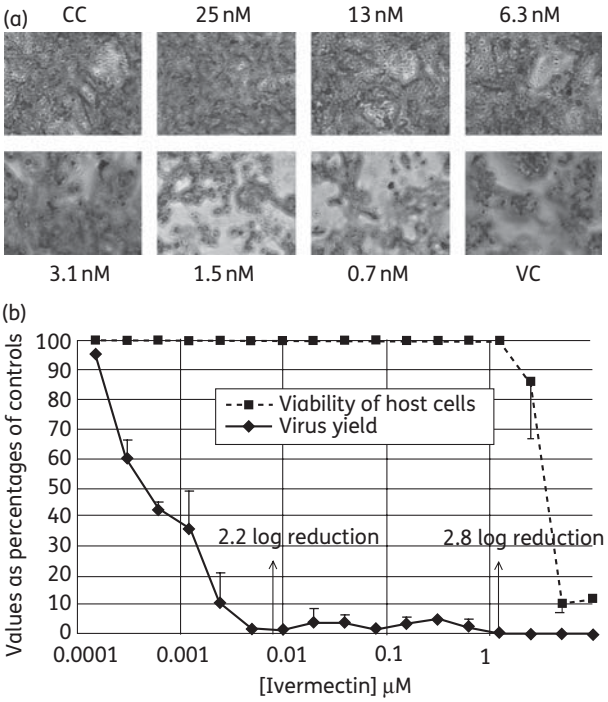


Figure 3. YFV-17D-induced CPE. (a) YFV-17D-induced CPE: concentration-dependent inhibition by ivermectin. At concentrations of >3 nM complete protection against virus-induced CPE formation (at 6 days post-infection) is observed. VC, virus-infected control without drug; CC, cell control (uninfected/untreated). Cells are shown at ×100 magnification. (b) Effect of ivermectin on the viability of uninfected host cells and on virus yield determined by RT-qPCR. Both results are reported as percentages of untreated controls. Arrows indicate the concentrations at which the reduction ('log reduction') in viral RNA levels was determined. Mean values of at least three independent experiments ± SD.

Despite the fact that ivermectin exerts *in vitro* anti-helicase activity (but neither anti-ATPase nor anti-RdRp activity), we cannot exclude the possibility that the molecule exerts its antiviral activity against flaviviruses via additional or other unrelated

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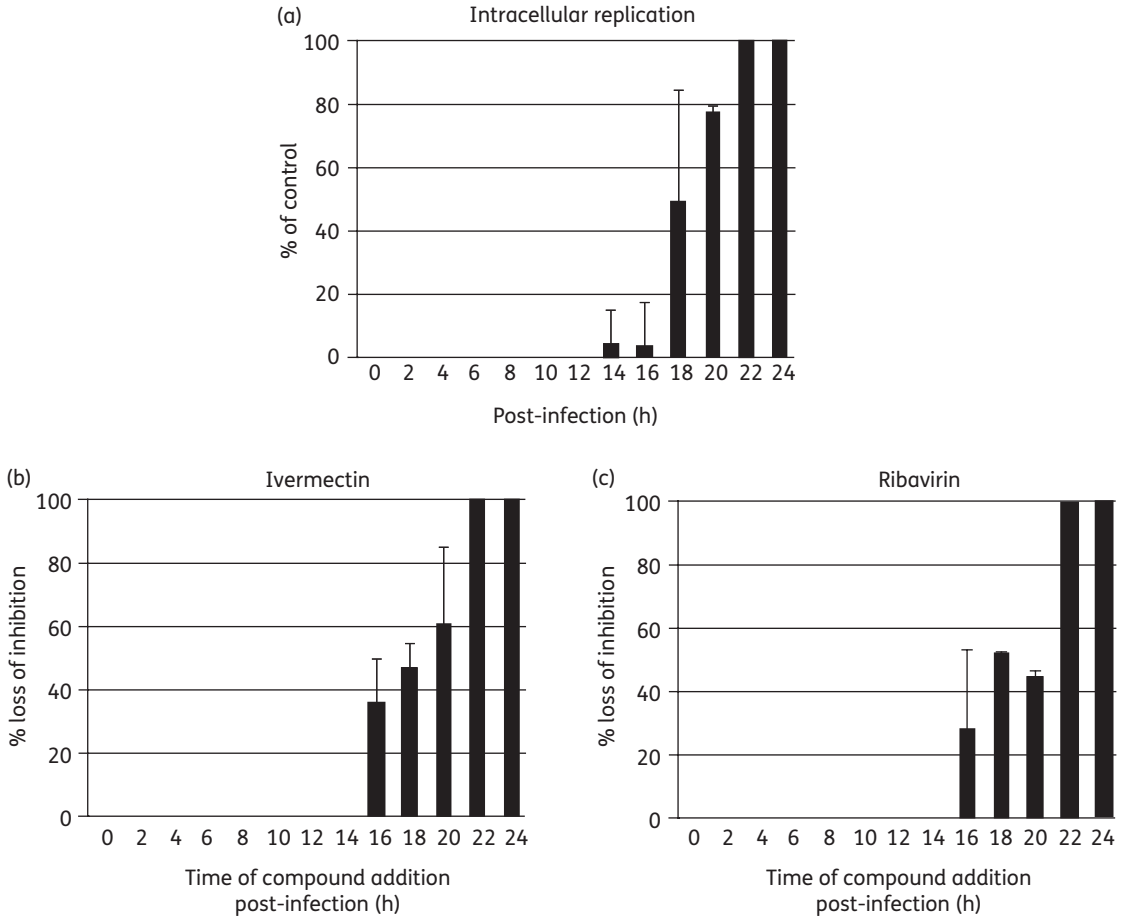


Figure 4. Time-dependent inhibition of YFV replication. (a) Kinetics of intracellular YFV-17D RNA synthesis following infection of Vero cells. Onset of viral RNA synthesis is detected at 14 h post-infection. (b) Effect of time of addition of ivermectin (left-hand panel) or ribavirin (right-hand panel) to YFV-17D-infected Vero cells. Both molecules gradually lost their protective activity when added at a timepoint (or later) that coincided with onset of viral replication in the control cultures.

mechanism(s). In particular for YFV, the net gap between the IC_{50} value against YFV helicase (120 nM) and the EC_{50} value in YFV cell cultures (0.5 nM) indicates that yet undiscovered process(es) other than helicase inhibition may contribute to the mechanism of action of ivermectin inhibition in cell culture. Besides, the mechanism behind the very selective and potent YFV activity may differ from that of the other flaviviruses (at least the ones we examined in our work), because of the different EC_{50} values observed in the three viruses. In fact, the lower EC_{50} value observed in DENV and, most strikingly, in WNV cell culture experiments could be related to different factors (i.e. compound solubility or permeability, metabolism, non-specific protein binding), including lower susceptibility of the viral replication complex (involving NS3 and NS5) with respect to the isolated helicase domain.

Our data, however, show that the mechanism of inhibition does not involve the early stages of the viral replication cycle (i.e. virus attachment and/or virus entry). The data presented nevertheless stress the key role played by helicase inhibition in the whole antiviral activity. To add independent proof that ivermectin acts in the infected cells by inhibition of the NS3 helicase, we tried to select ivermectin drug-resistant variants by serial passaging of YFV with increasing concentrations of the drug (expecting that adaptive mutations in the helicase domain would be selected). Unfortunately, even following extensive efforts, varying several experimental parameters (among others, multiplicity of infection, drug concentration increments and host cell type), drug-resistant virus variants were not selected after >30 serial passages of YFV, for >6 months in the presence of ivermectin. This may indicate that the barrier to resistance is high and that mutants may not emerge because they may not be viable.

Ivermectin is known as an anti-helminthic agent for oral administration. In the mid-1980s the compound was introduced as probably the most broad-spectrum anti-parasite medication ever,[43,44] which interferes with the parasite's nervous system and muscle function by binding and activating glutamate-gated chloride channels.[45] Ivermectin is used in humans mainly for the treatment of onchocerciasis, but is also effective against other worm infestations (such as strongyloidiasis, ascariasis, trichuriasis and enterobiasis). Ivermectin is also effective against most mites and some lice.

Considering that ivermectin has been used for the treatment of a variety of parasitic disease in man for >20 years, assessing its potential for the treatment of life-threatening flavivirus

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infections in clinical trials may require a minimum effort. Mining of epidemiological records in tropical regions where flaviviruses are endemic and where ivermectin has been administered for decades during population-wide onchocerciasis eradication programmes may offer first insights into the protective roles offered by the new application of this old drug.

Funding

This work was funded by the FP7 HEALTH-2010 Collaborative Project SILVER (No. 260644). A FEBS short-term fellowship also supported part of the activities reported here (M. P.).

Transparency declarations

None to declare.

Supplementary data

Figures S1, S2 and S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Editorial

Doxycycline as a potential partner of COVID-19 therapies

ARTICLE INFO

Article history:
Received 26 May 2020

Keywords:
COVID-19
Hydroxychloroquine
Doxycycline
2019 novel coronavirus
Remdesivir

ABSTRACT

Coronavirus disease 2019 (COVID-19) is a major public health challenge, and the current antiviral arsenal for treatment is limited, with questionable efficacy. Major efforts are under way for discovery of new effective agents, but the validation of new potential treatments for COVID-19 may take a long time. Therefore, the repurposing of existing drugs for new indications is needed. In this article, we argue for the potential benefits of using doxycycline with either hydroxycholoroquine or other putative agents for COVID-19 treatment, as doxycycline has antiviral and anti-inflammatory activities by dampening the cytokine storm and to prevent lung damage.

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Introduction

The 2019 novel coronavirus infection, dubbed COVID-19, has created an unprecedented public health crisis and threatened the lives of millions of people worldwide [1]. The immunopathogenesis of severe COVID-19 is partially understood and it is likely involves both a virus-driven damage and an exuberant host inflammatory response, both contributing to acute lung injury, acute respiratory distress syndrome (ARDS), and multiple organ failure [2]. Since there is no clear evidence of efficacy among available antivirals for COVID-19 and since the discovery and clinical testing of novel antiviral agents takes a long time, repurposing of existing drugs is of paramount importance. Priority should be given to drugs that combine antiviral and anti-inflammatory effects. In addition, candidate drugs should have an acceptable tolerability profile with no major adverse events or toxic effects.

Hydroxychloroquine with or without azithromycin

Several agents, including hydroxychloroquine have purported in vitro activity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-19 [3]. The anti-viral mechanism of hydroxychloroquine has not been entirely elucidated, but likely occurs through a change in pH at the surface of the cell membrane that inhibits the fusion and assembly of the virus [3]. Recent uncontrolled clinical data by Gautret et al. demonstrated that hydroxychloroquine treatment is associated with rapid virologic clearance, which occurred at day 6 in 70 % of patients on monotherapy versus in 12.5 % of patients on supportive care; adding azithromycin slightly enhanced this effect, with virologic clearance occurring at day 5 in 100 % of patients given the combination [4]. Despite the notable limitations of that study, including small number of patients, absence of randomization, selection bias for controls, and use of treatment in less-ill patients, this antimicrobial combination has generated a great deal of interest [4].

However, the hydroxychloroquine and azithromycin combination has raised major safety concerns, specifically, drug-drug interactions and cardiotoxicity, including fatal arrhythmia, particularly among infected elderly patients with underlying cardiopulmonary chronic illness [5]. The heightened risk of cardiotoxicity among older patients is of particular concern [6]. Also, patients with COVID-19 may develop infection-related cardiomyopathy (frequency is unknown) with direct and indirect cardiovascular complications, including acute myocardial injury, fulminant myocarditis (with a mortality rate up to 40 %–70 %), arrhythmias, and venous thromboembolism [5,7–9].

Antiviral effects of doxycycline

Given the risks of hydroxychloroquine and azithromycin in combination, we suggest hydroxychloroquine with doxycycline as a better alternative to azithromycin. Doxycycline and other tetracycline derivatives such as minocycline exhibit anti-inflammatory effects along with *in vitro* antiviral activity against several RNA viruses. Use of these agents have been associated with clinical improvement, even reversal of cytokine storm in some infections caused by RNA viruses, such as dengue fever [10].

The mechanism of the antiviral effects of tetracycline derivatives may be secondary to transcriptional upregulation of intracellular zinc finger antiviral protein (ZAP), an encoding gene in host cells [11,12]. ZAP can also bind to specific target viral mRNAs and represses the RNAs translation [13,14]. Experimental studies have used tetracycline to induce the overexpression of host ZAP in HEK293, rats and monkeys cell lines (Vero cells), which contributed to inhibition of RNA viruses such as the Dengue, Ebola, Human Immunodeficiency Virus, Zika, and Influenza A viruses [11,12,15–18].

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Also, in vitro studies have showed that doxycycline can repress Dengue virus infection in Vero cells through the inhibition of dengue serine protease enzymes and of viral entry [17,19]. Doxycycline showed the capacity to inhibit dengue virus replication in Vero cells culture and likely it interacts with the dengue virus E protein that is required for virus entry [19]. Similarly, doxycycline controls Chikungunya virus (CHIKV) infection through the inhibition of CHIKV cysteine protease of Vero cells and showed significant reduction of CHIKV blood titer of mice [20].

In addition, tetracycline derivatives such as doxycycline are highly lipophilic antimicrobials that chelate zinc compounds on matrix metalloproteinases (MMPs) of mammalian cells [21], and an in vitro study showed that murine coronaviruses rely on MMPs for cell fusion and viral replication [22]. Other mechanisms of viral fusion and replication by coronaviruses utilize host proteases [22], could be a possible target to doxycycline.

Anti-inflammatory effects of doxycycline

In COVID-19, elevated levels of blood interleukin (IL)-6 have been more commonly observed in severe COVID-19 illness and among non-survivors, suggesting that mortality might be due to virally-driven hyperinflammation and to cytokine storm [23]. Intense proinflammatory state has a central role in the pathogenesis of dengue and hemorrhagic fever, leading to cytokine storm [24]. Importantly, doxycycline reduced pro-inflammatory cytokines, including IL-6 and tumor necrosis factor (TNF)-α, in patients with dengue hemorrhagic fever, and the mortality rate was 46 % lower in the doxycycline-treated group (11.2 %) than in the untreated group (20.9 %) [24]. Moreover, doxycycline was more effective than tetracycline in the reduction of these pro-inflammatory cytokines [25].

Similarly, an in vitro study suggested that treatment with minocycline had dual anti-inflammatory effects and viral replication in cells infected with Enterovirus 71 infection, as minocycline reduced the viral cytopathic effect, viral protein expression, viral titers, levels of IL-6 and IL-8, and relative mRNA expression of TNF-α. Also, in a murine model, minocycline inhibited IL-6 and granulocyte colony-stimulating factor in plasma and TNF-α in the cerebellum [26].

In addition, severe acute respiratory syndrome–related coronavirus (SARS-CoV) encompasses a papain-like protease that significantly triggers an early growth response protein 1 (Egr-1)–dependent activation of transforming growth factor beta 1 (TGF-β1), resulting in upregulation of pro-fibrotic responses in vitro and in vivo in the lungs [27,28]. Recent computational methods study identified doxycycline among the drugs that could potentially be used to inhibit SARS-CoV-2 papain-like protease [29].

Severe COVID-19, ARDS, and pathophysiologic and therapeutic considerations

Respiratory failure from ARDS is the leading cause of mortality in COVID-19 patients [30]. Various pro-inflammatory cytokines and chemokines, including IL-6, TNF-α, and profibrotic factors (*TGFB1, CCN2,* and *PDGFA*), are also implicated in tissue damage and vascular leakage and can stimulate pulmonary fibrosis in SARS-CoV infection [31]. The pathologic features of COVID-19 closely resemble those of SARS-CoV infection, which causes massive lung tissue remodeling through the urokinase, coagulation, and wound-healing pathways and through extracellular matrix proteins, including MMPs [31]. MMPs are involved in lung remodeling and destruction of the extracellular matrix, leading to damage of the endothelial basal lamina and increased vascular permeability [32,33]. Importantly, mechanical ventilation, which has a primary role in ARDS management, is associated with further lung injury through activation of MMPs, leading to ventilation-induced lung injury [34].

As mentioned earlier, doxycycline is a strong and broad-spectrum inhibitor of MMPs (a family of more than 24 zinc-dependent proteases). Furthermore, experimental studies have showed that treatment with doxycycline conferred a protective role in lung injury [35,36]. A prophylactic use of doxycycline in mice infected with virulent influenza H3N2 virus attenuates the occurrence of acute lung injury [37]. The tetracycline class of antimicrobials overall has proven a clinically useful tool in MMP inhibition through their ability to chelate the catalytic Zn^{2+} ion, which is essential for MMP activity, independently of their antimicrobial properties [34]. Among the tetracycline derivatives, doxycycline is the most potent MMP inhibitor, even at a subantimicrobial dose (25 mg) [34]. As lung immune injury/ARDS is prominent in patients with severe COVID-19, inhibiting MMPs may help repair the damaged lung tissue and enhance recovery [38].

Future study design considerations

In light of these potential benefits, we propose the use of doxycycline (preferably) or minocycline as a partner agent with hydroxychloroquine or with other promising antiviral COVID-19 therapies such as remdesivir, particularly in elderly patients with multiple health conditions, especially cardiac comorbidities. Doxycycline is usually prescribed as a part of empiric treatment for atypical bacterial pneumonia or community-acquired pneumonia based on the recent evidence-based clinical practice guidelines [39]. Therefore, it may be useful to conduct a large retrospective cohort study assessing disease severity, co-infections, mortality rate, length of hospitalization, and the need for invasive ventilation among COVID-19–infected patients who received a doxycycline-based or other tetracycline-based empiric antimicrobial regimen.

In addition, placebo-controlled randomized clinical trials divided into two arms (treatment arm, antiviral (i.e. remdesivir) plus doxycycline versus the control arm, antiviral plus placebo) and should enroll COVID-19 patients regardless of the severity of illness at presentation. Primary end points, in addition to mortality rate, should include clinical improvement (defervescence), progression to respiratory failure, need for mechanical ventilation (duration and extubation), virologic clearance, and length of stay at the hospital.

Conclusion

Because patients with COVID-19 are in need of both antiviral and anti-inflammatory treatment as well as protection against lung damage, studies of proposed combination therapy is warranted. As doxycycline is inexpensive and widely available, has a safe tolerability profile, and is an attractive option for the treatment of COVID-19 as well as potentially alleviating the lung sequelae and also providing coverage against atypical bacterial pneumonia such as *Mycoplasma* pneumoniae and *Legionella pneumophilia*.

Funding

No funding of any kind has been received.

CRediT authorship contribution statement

Alexandre E. Malek: Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Bruno P. Granwehr:** Writing - original draft. **Dimitrios P. Kontoyiannis:** Conceptualization, Supervision, Writing - review & editing.

FOI 26-3132 - Document 5

Declaration of Competing Interest

None of the authors have any financial or personal relationships with other people or organizations that might pose a conflict of interest in connection with the submitted article.

Acknowledgments

We acknowledge Sarah Bronson in Scientific Publications, Research Medical Library, The University of Texas MD Anderson Cancer Center, for editing the manuscript.

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Received 26 May 2020

ChemRxiv™
doi.org/10.26434/chemrxiv.12630539.v1
EFTA26-3/32 - Document 2

A Combination of Ivermectin and Doxycycline Possibly Blocks the Viral Entry and Modulate the Innate Immune Response in COVID-19 Patients

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Submitted date: 09/07/2020 • Posted date: 09/07/2020

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Citation information: Maurya, Dharmendra Kumar (2020): A Combination of Ivermectin and Doxycycline Possibly Blocks the Viral Entry and Modulate the Innate Immune Response in COVID-19 Patients. ChemRxiv. Preprint. https://doi.org/10.26434/chemrxiv.12630539.v1

The current outbreak of the corona virus disease 2019 (COVID-19), has affected almost entire world and become pandemic now. Currently, there is neither any FDA approved drugs nor any vaccines available to control it. Very recently in Bangladesh, a group of doctors reported astounding success in treating patients suffering from COVID-19 with two commonly used drugs, Ivermectin and Doxycycline. In the current study we have explored the possible mechanism by which these drugs might have worked for the positive response in the COVID-19 patients. To explore the mechanism we have used molecular docking and molecular dynamics simulation approach. Effectiveness of Ivermectin and doxycycline were evaluated against Main Protease (Mpro), Spike (S) protein, Nucleocapsid (N), RNA-dependent RNA polymerase (RdRp, NSP12), ADP Ribose Phosphatase (NSP3), Endoribonuclease (NSP15) and methyltransferase (NSP10-NSP16 complex) of SARS-CoV-2 as well as human angiotensin converting enzyme 2 (ACE2) receptor. Our study shows that both Ivermectin and doxycycline have significantly bind with SARS-CoV-2 proteins but Ivermectin was better binding than doxycycline. Ivermectin showed a perfect binding site to the Spike-RBD and ACE2 interacting region indicating that it might be interfering in the interaction of spike with ACE2 and preventing the viral entry in to the host cells. Ivermectin also exhibited significant binding affinity with different SARS-CoV-2 structural and non-structural proteins (NSPs) which have diverse functions in virus life cycle. Significant binding of Ivermectin with RdRp indicate its role in the inhibition of the viral replication and ultimately impeding the multiplication of the virus. Ivermectin also possess significant binding affinity with NSP3, NSP10, NSP15 and NSP16 which helps virus in escaping from host immune system. Molecular dynamics simulation study shows that binding of the Ivermectin with Mpro, Spike, NSP3, NSP16 and ACE2 was quiet stable. Thus, our docking and simulation studies reveal that combination of Ivermectin and doxycycline might be executing the effect by inhibition of viral entry and enhance viral load clearance by targeting various viral functional proteins.

File list (1)

Ivermectin+Doxycycline MS.pdf (1.88 MiB) view on ChemRxiv • download file

A Combination of Ivermectin and Doxycycline Possibly Blocks the Viral Entry and Modulate the Innate Immune Response in COVID-19 Patients

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Abstract

The current outbreak of the corona virus disease 2019 (COVID-19), has affected almost entire world and become pandemic now. Currently, there is neither any FDA approved drugs nor any vaccines available to control it. Very recently in Bangladesh, a group of doctors reported astounding success in treating patients suffering from COVID-19 with two commonly used drugs, Ivermectin and Doxycycline. In the current study we have explored the possible mechanism by which these drugs might have worked for the positive response in the COVID-19 patients. To explore the mechanism we have used molecular docking and molecular dynamics simulation approach. Effectiveness of Ivermectin and doxycycline were evaluated against Main Protease (Mpro), Spike (S) protein, Nucleocapsid (N), RNA-dependent RNA polymerase (RdRp, NSP12), ADP Ribose Phosphatase (NSP3), Endoribonuclease (NSP15) and methyltransferase (NSP10-NSP16 complex) of SARS-CoV-2 as well as human angiotensin converting enzyme 2 (ACE2) receptor. Our study shows that both Ivermectin and doxycycline have significantly bind with SARS-CoV-2 proteins but Ivermectin was better binding than doxycycline. Ivermectin showed a perfect binding site to the Spike-RBD and ACE2 interacting region indicating that it might be interfering in the interaction of spike with ACE2 and preventing the viral entry in to the host cells. Ivermectin also exhibited significant binding affinity with different SARS-CoV-2 structural and non-structural proteins (NSPs) which have diverse functions in virus life cycle. Significant binding of Ivermectin with RdRp indicate its role in the inhibition of the viral replication and ultimately impeding the multiplication of the virus. Ivermectin also possess significant binding affinity with NSP3, NSP10, NSP15 and NSP16 which helps virus in escaping from host immune system. Molecular dynamics simulation study shows that binding of the Ivermectin with Mpro, Spike, NSP3, NSP16 and ACE2 was quiet stable. Thus, our docking and simulation studies reveal that combination of Ivermectin and doxycycline might be executing the effect by inhibition of viral entry and enhance viral load clearance by targeting various viral functional proteins.

Keywords: COVID-19; SARS-CoV-2; Ivermectin; Doxycycline; RNA dependent RNA polymerase; Molecular docking; Molecular dynamics simulation

Introduction

The current outbreak of the corona virus disease 2019 (COVID-19), has affected almost entire World and become pandemic. Because of its high rate of transmission and infectivity, World Health Organization (WHO) has declared it as a global disaster. Different therapeutics are currently being evaluated in clinical trials but there is no specific drug or vaccine that has been proven its effectiveness to date. Most of the current treatment options against COVID-19 are based on previous experiences/ lessons gain from the SARS-CoV and MERS-CoV epidemics. Like other CoVs, SARS-CoV-2 has a positive-sense single-stranded RNA as genome and four structural proteins, namely the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. As for mechanism of the CoVs infection is concerned, similar to other CoVs, it uses their surface spike proteins and interact with its cognate receptor i.e angiotensin-converting enzyme 2 (ACE2) receptor present on the host cell surface. Unlike, other CoVs, SARS-CoV-2 spike protein has high affinity to the host cellular ACE2 receptor (Zhang, Penninger, Li, Zhong & Slutsky 2020). Most of the current research for the development of effective therapeutics

against SARS-VCoV-2 are based on the screening of existing broad-spectrum anti-virals, small molecule libraries, FDA approved drug libraries and traditional alternative medicines (Maurya 2020; Maurya & Sharma 2020; Wu et al. 2020; Zhou et al. 2020). Very recently, a team of the doctors from Bangladesh Medical College Hospital, has reported that Ivermectin given single dose with doxycycline, yielded the near-miraculous results in curing COVID-19 patients.

Ivermectin is a FDA-approved broad spectrum anti-parasitic drug (González Canga et al. 2008). It affects a plethora of invertebrate species, including parasitic arachnids, nematodes and insects. It has been earlier shown to exert anti-viral activity in vitro against Dengue fever virus (DENV), influenza virus, West Nile Virus and Venezuelan equine encephalitis virus (Lundberg et al. 2013; Tay et al. 2013; Götz et al. 2016). Very recently, Caly et al (2020) have shown the efficacy of the Ivermectin in Vero-hSLAM cells infection with SARS-CoV-2 and observed an effective loss of viral material within 48h (Caly, Druce, Catton, Jans & Wagstaff 2020). Based on their finding, Caly et al (2020) hypothesised that it might we working by inhibiting Importin α/β1 (IMPα/β1)-mediated nuclear import of viral proteins, as shown for other RNA viruses (Caly, Wagstaff & Jans 2012; Lundberg et al. 2013; Tay et al. 2013; Caly et al. 2020; Yang et al. 2020). As for safety is concerned, Ivermectin has an established safety profile for human use (González Canga et al. 2008; Buonfrate et al. 2019). The second drug, doxycycline is a synthetic antibiotic derived from tetracycline and have broad spectrum antibiotic property. Doxycycline other than antibiotics, are known to inhibit M2-type polarization of macrophages (He & Marneros 2014). Doxycycline is a FDA approved antibiotic since 1967 and is extensively referred by most clinicians. As for its safety is concerned, doxycycline has a long history of safety.

In the current study, we have explored the possible mechanisms to explain the above experimental and clinical findings using molecular docking and molecular dynamics simulation studies. For this, Ivermectin and doxycycline were docked with different viral proteins (such as spike, Mpro, PLpro, RNA dependent RNA polymerase, Nucleocapsid, NSP3, NSP10, NSP15 and NSP16) and host cell receptor (ACE2). Our molecular docking study predicted that Ivermectin and doxycycline both have significant binding affinity for SARS-CoV-2 spike and ACE2 interacting region, indicating poor interaction for viral entry inside the cells. The binding affinity of the Ivermectin is much better than the doxycycline. At the same time, Ivermectin has significantly high binding affinity with the viral RNA dependent RNA polymerase (RdRp, NSP12), indicating poor viral genome replication for viral multiplication. Ivermectin significantly inhibited ADP Ribose Phosphatase (NSP3), Endoribonuclease (NSP15) and methyltransferase (NSP10-NSP16 complex) of SARS-CoV-2 which were involved in the virus escape from the host innate immune system. Ivermectin and doxycycline both bind with SARS-CoV-2 main protease (Mpro) adjacent to its active site. Our study provides the possible mechanism of the action of this combination and a scientific justification for their application in prevention and management of COVID-19 crisis.

Materials and Methods

Chemical structure of molecules and target proteins: Chemical structures information for Ivermectin and doxycycline were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov). In order to study the mode of interaction of Ivermectin

and doxycycline with various SARS-CoV-2 proteins and receptors found on host cells, following PDB ID's 6lu7 (SARS-CoV-2 Mpro), 6m0j (SARS-CoV-2 spike), 6m71 (SARS-CoV-2 RdRp, NSP12), 6w02 (NSP3), 6w9c (PLpro), 6m3m (Nucleocapsid), 6w4b (NSP9), 6wkq (NSP10), 6vww (NSP15), 6wkq (NSP16) and 6m0j (ACE2) were used. All the protein structures were retrieved from protein data bank (https://www.rcsb.org/) and cleaned using USCF Chimera software (Pettersen et al. 2004).

Molecular docking: PyRx virtual screening tool was used for preparation of the ligands and receptor to perform the molecular docking. Molecular docking was performing using Vina wizard of PyRx virtual screening tool (Dallakyan & Olson 2015). For preparation of protein input files, all the water molecules, ligands and ions were removed from *.pdb files and polar hydrogens were added before saving *.pdbqt format. Both the molecules were energy minimized and saved in *.pdbqt format after adding polar hydrogens for further docking process. Following AutoDock Vina docking parameters such as (center_x = -26.2834, center_y = 12.5988, center_z = 58.9648, size_x = 66.124, size_y = 22.9423, size_z = 51.456), (center_x = -32.483, center_y = 26.077, center_z = 7.923, size_x = 52.974, size_y = 46.699, size_z = 30.699) and (center_x = -25.0997, center_y = 19.903, center_z = 3.047, size_x = 78.872, size_y = 67.603, size_z = 35.866) were used for SARS-CoV-2 Mpro (PDB ID: 6LU7), SARS-CoV-2 spike (PDB ID: 6m0j) and human ACE2 (PDB ID: 6m0j) respectively. For docking RdRp (PDB ID: 6m71) NSP7 and NSP8 was removed from the NSP12 and grid box for docking parameter was set at the NSP12-NSP7 and NSP12-NSP8 interface. Docking parameters used for NSP12 were center_x = 112.289, center_y = 131.245, center_z = 141.597, size_x = 83.597, size_y = 69.959, size_z = 45.240. A blind docking was performed for the remaining target receptor proteins.

Interactions of the ligand with critical residues: 2D interaction of docked complex were analysed for the visualization of the best binding poses. As the importance of hydrogen bonding is well documented in drug discovery and design, further hydrogen bond and other non-polar interactions of best binding poses were examined using Discovery studio software (Dassault Systèmes BIOVIA, San Diego, 2016).

Molecular dynamics simulation: The protein-ligand complex for spike, Mpro, NSP3, NSP16 and human ACE2 receptor with Ivermectin were obtained after the molecular docking study. The simulation systems for protein-ligand complex without or with the Ivermectin were performed using the GROMACS software (Abraham et al. 2015). Protein topology was prepared using pdb2gmx module of GROMACS. Whereas ligand topology was derived from Prodrg server (http://prodrg1.dyndns.org/)(van Aalten et al. 1996). MD simulation was performed with Gromos53a6.ff force-field using the GROMACS. Proteins were solvated using SPC (simple point charge) water model and counter ions were added to neutralize the system. After that, system was energy minimized and further water and ions were allowed to equilibrate around the protein in a two-step equilibration process. The first part of equilibration was at a constant number of particles, volume, and temperature (NVT). The second part of equilibration was at a constant number of particles, pressure, and temperature (NPT). NVT and NPT equilibration simulations were performed for 100 ps. Following

equilibration, the production simulation duration was performed for 5000 picoseconds (ps). The trajectory data were saved at different time points to analyse the change in the dynamics of protein-ligand complex. After completion of MD simulation, results for flexibility for protein-ligand complexes were analysed using root mean square deviation (RMSD) and root mean square fluctuation (RMSF) using GROMACS.

Results and Discussion

Different therapeutics have different targets for the prevention of the CoVs severity (Prajapat et al. 2020). Some may block virus spike interaction with host cell receptors, or some may interfere with viral genome replication and some other may obstruct the process of virus assembly (Prajapat et al. 2020). In the current study, drugs Ivermectin and doxycycline were evaluated for their binding affinities against SARS-CoV-2 spike, Mpro, PLpro, RdRp, nucleocapsid, NSP3, NSP9, NSP10, NSP15, NSP16 and human ACE2 receptor by molecular docking. Further binding stability of spike, Mpro, NSP3, NSP16 and ACE2 protein complexed with Ivermectin were evaluated by molecular dynamics simulation.

Molecular docking: Our molecular docking study revealed that both Ivermectin and doxycycline have significant binding affinity with various SARS-CoV-2 proteins i.e Mpro, spike, PLpro, RdRp, nucleocapsid, NSP3, NSP9, NSP10, NSP15, NSP16 and host ACE2 receptor (Table 1). Ivermectin showed better binding affinity with these target proteins compared to doxycycline. During SARS-CoV-2 infection, interaction of spike-RBD protein with the host cell receptor facilitate virus invasion and determine viral tissue or host tropism (Li 2016). Initial interactions of spike with host receptor (ACE2), and subsequent fusion of the host and viral membrane allows the viral genome to enter inside the host cells (Li 2016). It is now well established that, amino acid residues SER19, GLN24, THR27, PHE28, ASP30, LYS31, HIS34, GLU35, GLU37, ASP38, TYR41, GLN42, LEU45, LEU79, MET82, TYR83, ASN330, LYS353, GLY354, ASP355 and ARG357 of the human ACE2 interact with amino acid residues **LYS417,** GLY446, **TYR449, TYR453**, **LEU455, PHE456,** TYR473, ALA475, GLY476, GLU484, PHE486, ASN487, TYR489, PHE490, GLN493, **GLY496, GLN498,** THR500, ASN501, GLY502 and **TYR505** of SARS-CoV-2 spike-RBD protein (Wang et al. 2020). Our results show that, Ivermectin binds at the junction of spike-RBD and ACE2 interaction site indicating that it might have potential to inhibit the entry of the virus to the host cell (Fig 1a). Different amino acid residues of spike protein interacting with Ivermectin are ARG403, ASP405, ARG408, GLN409, GLY416, **LYS417, TYR449, TYR453, LEU455, PHE456,** SER494, TYR495, **GLY496, GLN498** and **TYR505** whereas amino acid interacting with ACE2 includes LYS26, LEU29, **ASP30,** ASN33 **HIS34, GLU37,** THR92, VAL93, GLN96, ALA386, ALS387, GLN388, PRO389, LEU392, ARG393, ARG559 and SER563. From the Figure 1A, it is clear that Ivermectin independently bind at the interacting region of viral spike and host ACE2 receptor indicating that it might hamper the interaction of spike and ACE2 receptor and inhibit the virus entry inside the cells. Figure 1B, shows the 2D interaction of Ivermectin and doxycycline with different protein residues of spike and ACE2. The

predicted binding energies of Ivermectin and doxycycline are -7.2 and -6.6 kcal/mol for spike protein and -7.5 and -7.6 kcal/mol for ACE2 receptor respectively.

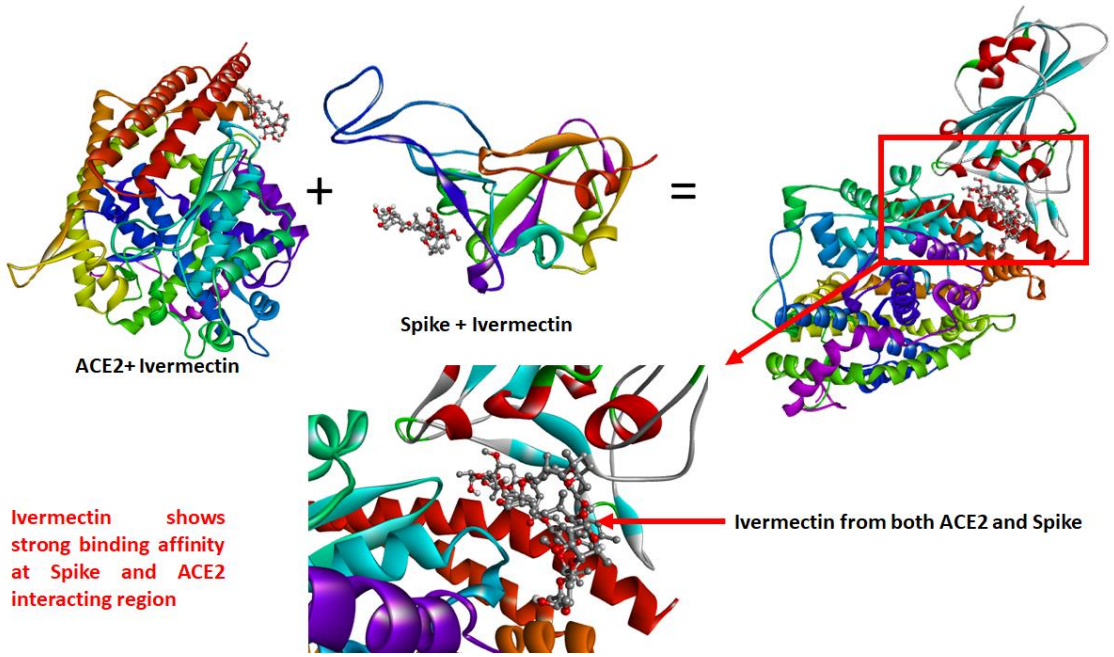
Table 1: *Predicted binding energy and inhibition constant of Ivermectin and Doxycycline for various SARS-CoV-2 target proteins and human ACE2 receptor*

Drugs	Targets, PDB ID's	Predicted binding energy (kcal/mol)	Predicted Inhibition constant, Ki (μM)
Ivermectin	Mpro, 6lu7	-8.4	0.69
	PLpro, 6w9c	-8.5	0.58
	Spike, 6m0j	-7.2	5.21
	Nucleocapsid, 6m3m	-8.2	0.96
	NSP3, 6w02	-8.0	1.35
	NSP9, 6w4b	-7.9	1.60
	NSP10, 6wkq	-8.9	0.69
	NSP12, 6m71	-9.1	0.21
	NSP15, 6vww	-8.4	0.69
	NSP16, 6wkq	-8.3	0.81
	ACE2, 6m9j	-7.5	3.14
Doxycycline	Mpro, 6lu7	-7.1	6.16
	PLpro, 6w9c	-7.1	6.16
	Spike, 6m0j	-6.6	14.35
	Nucleocapsid, 6m3m	-6.3	23.82
	NSP3, 6w02	-7.1	6.16
	NSP9, 6w4b	-6.1	33.40
	NSP10, 6wkq	-6.9	8.64
	NSP12, 6m71	-7.9	1.60
	NSP15, 6vww	-7.5	3.14
	NSP16, 6wkq	-7.7	2.24
	ACE2, 6m9j	-7.6	2.65

The RdRp is the fundamental component of SARS-CoV-2 replication/transcription machinery. Figure 2, shows the predicted binding site and 2D interaction of Ivermectin and doxycycline for SARS-CoV-2 RdRp. The anticipated binding energies of Ivermectin and doxycycline for RdRp are -9.1 and -7.9 kcal/mol respectively. SARS-CoV-2 Mpro is considered as a promising druggable target because it plays vital role in viral gene expression and replication and are essential for maturation cleavage events of viral precursor polyprotein. The critical maturation cleavage events within the viral precursor polyprotein are mediated by the Mpro. Figure 3, shows the predicted binding site and 2D interaction of Ivermectin and doxycycline with SARS-CoV-2 Mpro. The predicted binding energies of Ivermectin and doxycycline for Mpro are -8.4 and -7.1 kcal/mol respectively. Our study shows that although both Ivermectin and doxycycline have strong binding affinity with the Mpro, but they bind off-site to the active site of the Mpro indicating they may not directly inhibit Mpro rather will decrease their activity by interfering with its flexibility (Figure 3).

Figure 1: *Interaction of Ivermectin and doxycycline with SARS-CoV-2 spike and human ACE2 receptor*

A. *Interaction of Ivermectin with SARS-CoV-2 spike and human ACE2 receptor.*



B. *2D interaction of Ivermectin and doxycycline with SARS-CoV-2 spike and human ACE2 receptor.*

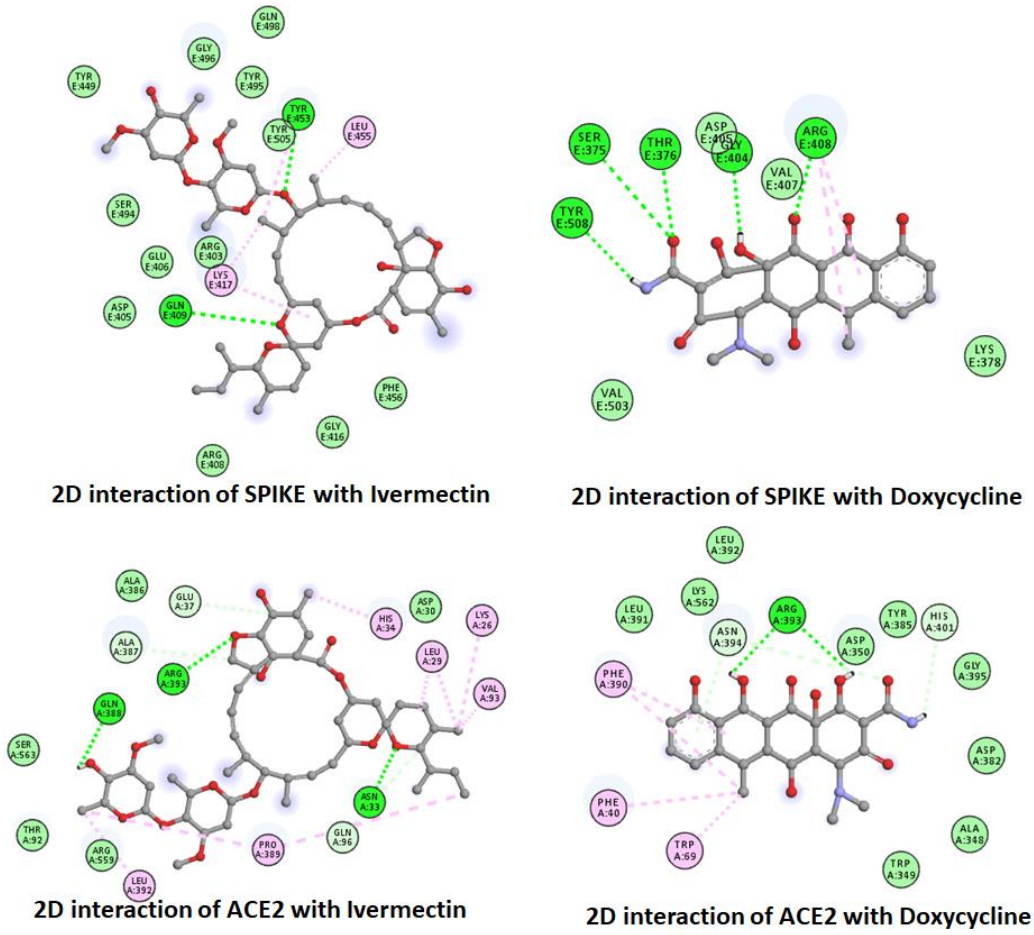
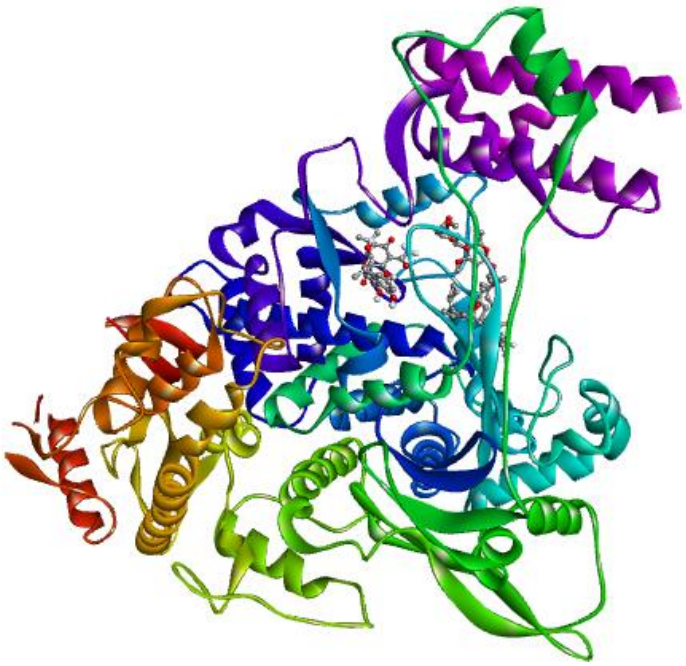


Figure 2: *Interaction of Ivermectin and doxycycline with SARS-CoV-2 RdRp.*

A. *Binding site of Ivermectin and doxycycline with SARS-CoV-2 RdRp.*



B. *2D interaction of Ivermectin and doxycycline with SARS-CoV-2 RdRp.*

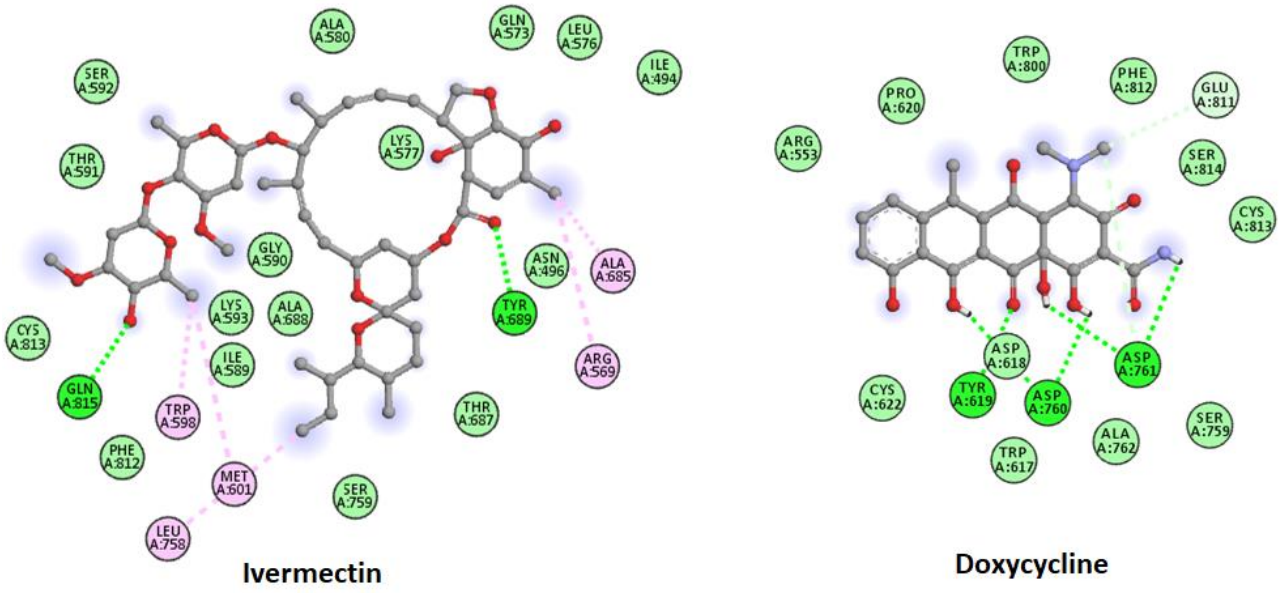
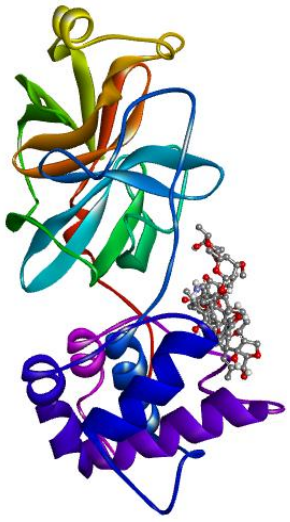
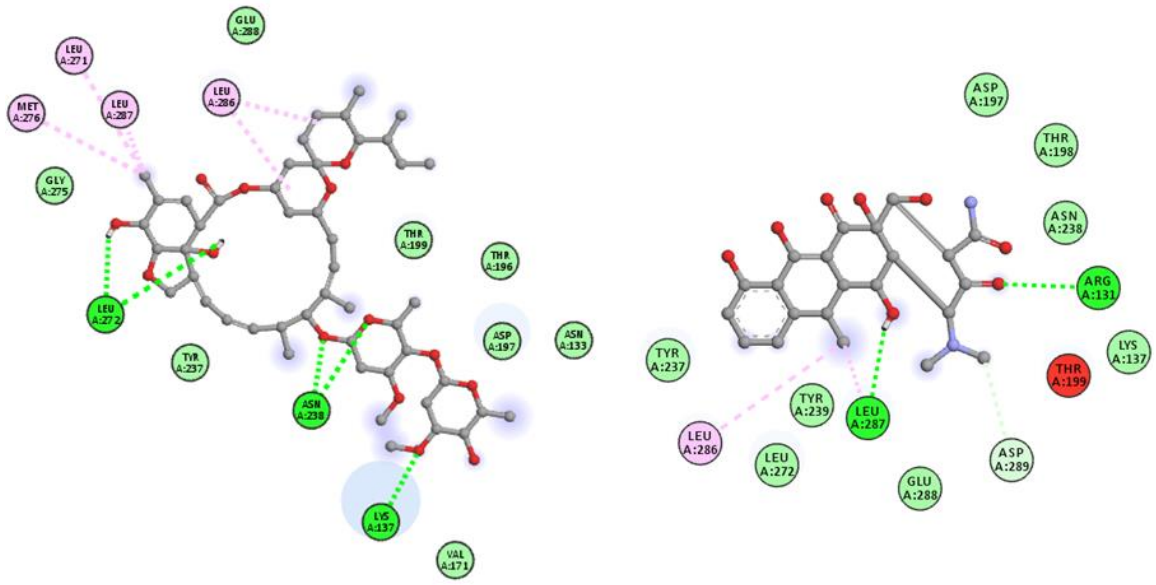


Figure 3. *Interaction of Ivermectin and doxycycline with SARS-CoV-2 Mpro.*

A. *Binding site of Ivermectin and doxycycline with SARS-CoV-2 Mpro.*



B. *2D interaction of Ivermectin and doxycycline with SARS-CoV-2 Mpro.*



Ivermectin **Doxycycline**

The NSP3 is the largest multi-domain protein encoded by the CoVs and is a vital component of the replication/transcription complex (RTC). NSP3 function as an ADP-ribose-1'-phosphatase. Besides its macrodomain, NSP3 also contains a papain-like protease (PLpro) required during the processing of the CoVs NSPs. Ubiquitination is a posttranslational modification that, among many other functions, is very important in the regulation of innate immune pathways (Mielech, Chen, Mesecar & Baker 2014). PLpro is believed to antagonize the IFN response through its de-ubiquitinating enzyme activity, which allows the protease to remove ubiquitin from a substrate. Figure 4, shows the predicted binding site and 2D interaction of Ivermectin and doxycycline with SARS-CoV-2 NSP3 and PLpro. The predicted binding energies of Ivermectin and doxycycline -8.0 and -7.1 kcal/mol for NSP3 whereas -8.5 and -7.1 kcal/mol for PLpro. These observations show that, binding of Ivermectin with NSP3 and PLpro may block its enzymatic activity and thereby prevent SARS-CoV-2 from down-regulating the anti-viral interferon response. This may improve viral load clearance by host innate immune system and reduce the extent of viral pathogenesis in COVID-19 patients without any previous memory of the antigen.

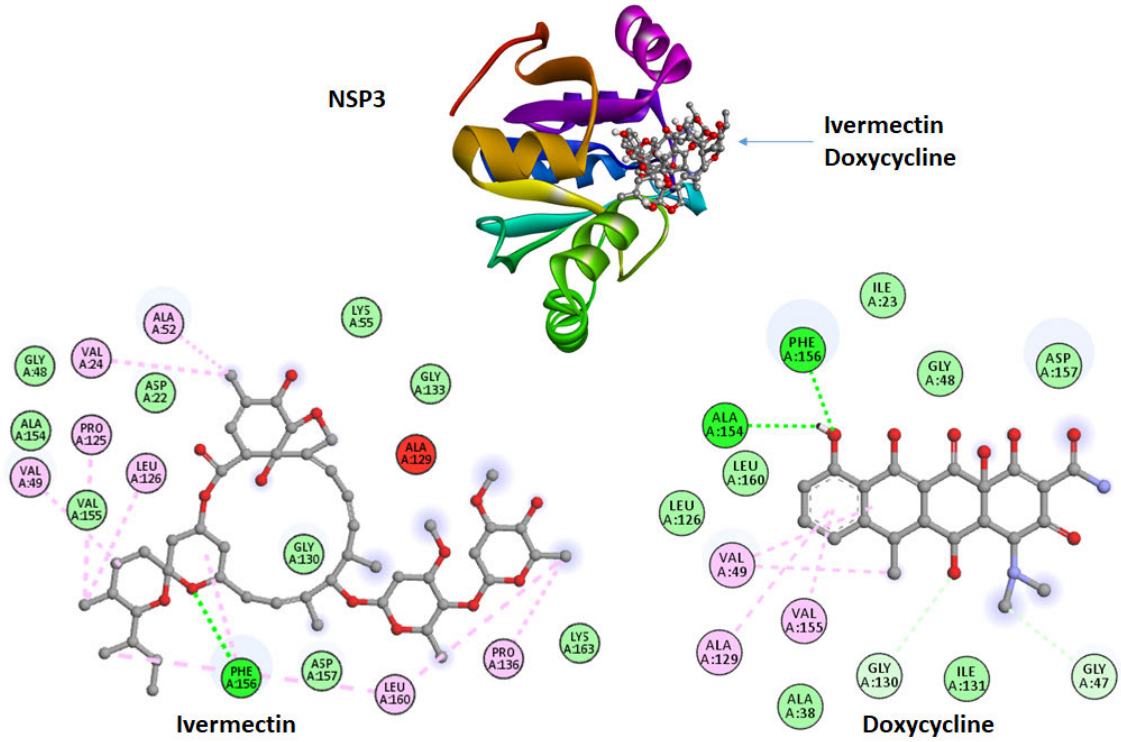
Viral RNAs are capped to safeguard their stability, efficient translation, and evading the innate immune system of the host cell. The RNA capping tactic are employed by several RNA viruses to avoid immune detection by toll-like receptor 7 (TLR7) and toll-like receptor 8 (TLR8) (Hyde & Diamond 2015). The viral RNA capping machinery is anatomically and mechanistically different compared to eukaryotic mRNA capping system. The CoVs NSP16 protein is a RNA cap modifying enzyme and become active when complexed with its activating partner NSP10 (Decroly et al. 2011). NSP16 binds with NSP10 resulting a complex which exhibits RNA cap (nucleoside-2'-O)-methyltransferase activity. Figure 5, shows the predicted binding site and 2D interaction of Ivermectin and doxycycline with SARS-CoV-2 NSP16 and NSP10. Our study

shows that Ivermectin binds to the S-Adenosylmethionine binding site of NSP16 with a binding affinity of -8.3 kcal/mol. It also binds with the NSP16 cognate activation partners NSP10 with a binding affinity of -8.9 kcal/mol. This indicates that the binding of Ivermectin to the S-Adenosylmethionine binding site will hamper the essential methyl-group transfer and inhibit the methyltransferase activity of NSP16. In the absence of methyl group attachment to the 5'cap of viral RNA, it will get easily detected by host innate immune system. Strong binding affinity of Ivermectin with NSP10 may also interfere in the formation of NSP16-NSP10 complex.

Some of the other important CoVs proteins such as nucleocapsid (N), NSP9 and NSP15 were also used for molecular docking study. The CoVs N protein is a structural protein that forms complexes with viral genome, interacts with the viral membrane protein during virion assembly and plays an important role in enhancing the efficiency of virus transcription and assembly (McBride, van Zyl & Fielding 2014). N-terminal region of nucleocapsid protein consists of positively charged amino acids, which are responsible for its RNA binding. CoVs NSP9 is most likely involved in viral RNA synthesis. NSP9 binds with RNA and interacts with NSP8 to execute its essential functions (Sutton et al. 2004). Dimeric form of NSP9 is important

Figure 4. *Interaction of Ivermectin and doxycycline with SARS-CoV-2 NSP3 and PLpro.*

A. *Binding site of Ivermectin and doxycycline with SARS-CoV-2 NSP3 and their 2D interactions.*



B. *Binding site of Ivermectin and doxycycline with SARS-CoV-2 PLpro and their 2D interactions.*

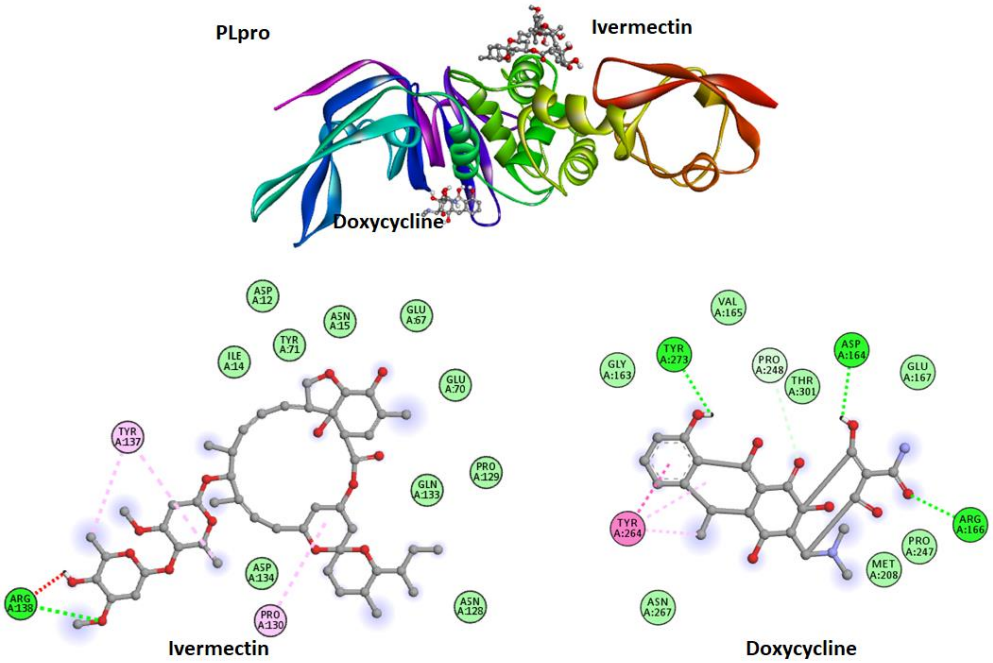
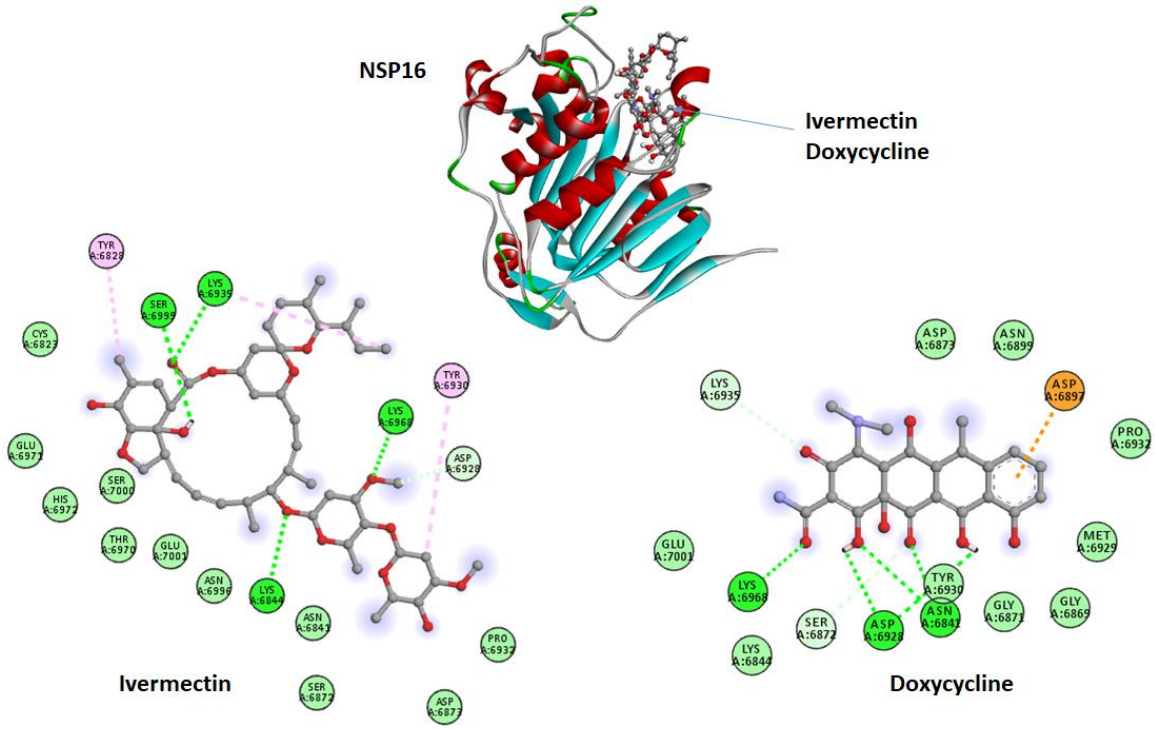
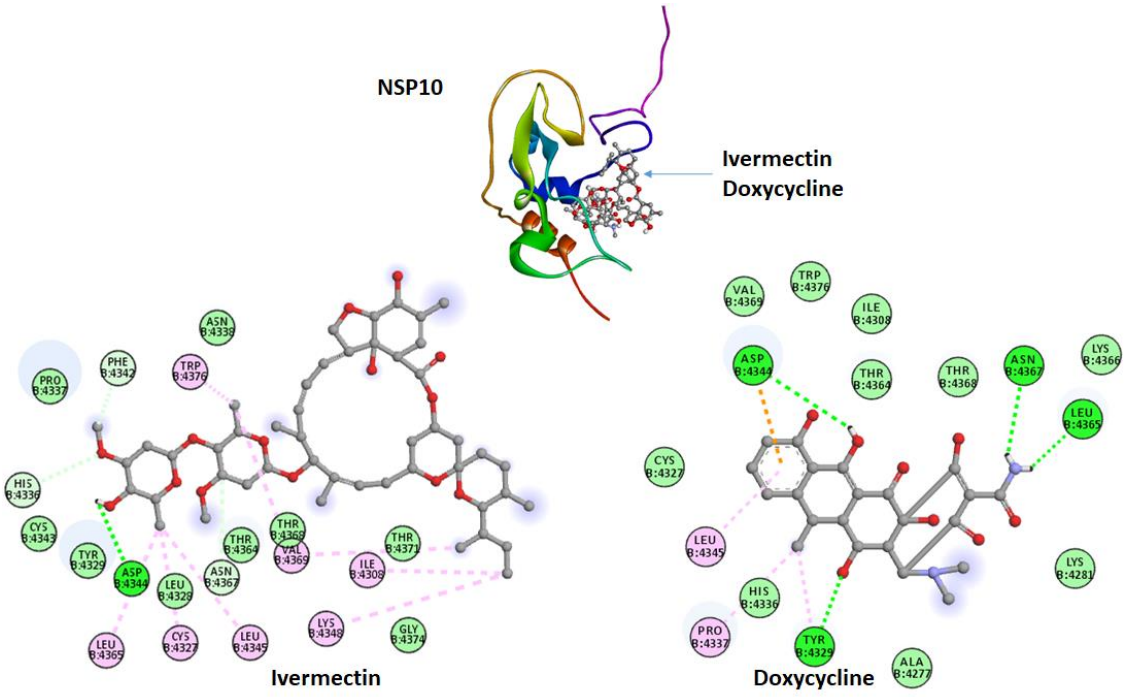


Figure 5. *Interaction of Ivermectin and doxycycline with SARS-CoV-2 NSP16 and NSP10.*

A. *Binding site of Ivermectin and doxycycline with SARS-CoV-2 NSP16 and their 2D interactions.*



B. *Binding site of Ivermectin and doxycycline with SARS-CoV-2 NSP16 and their 2D interactions.*



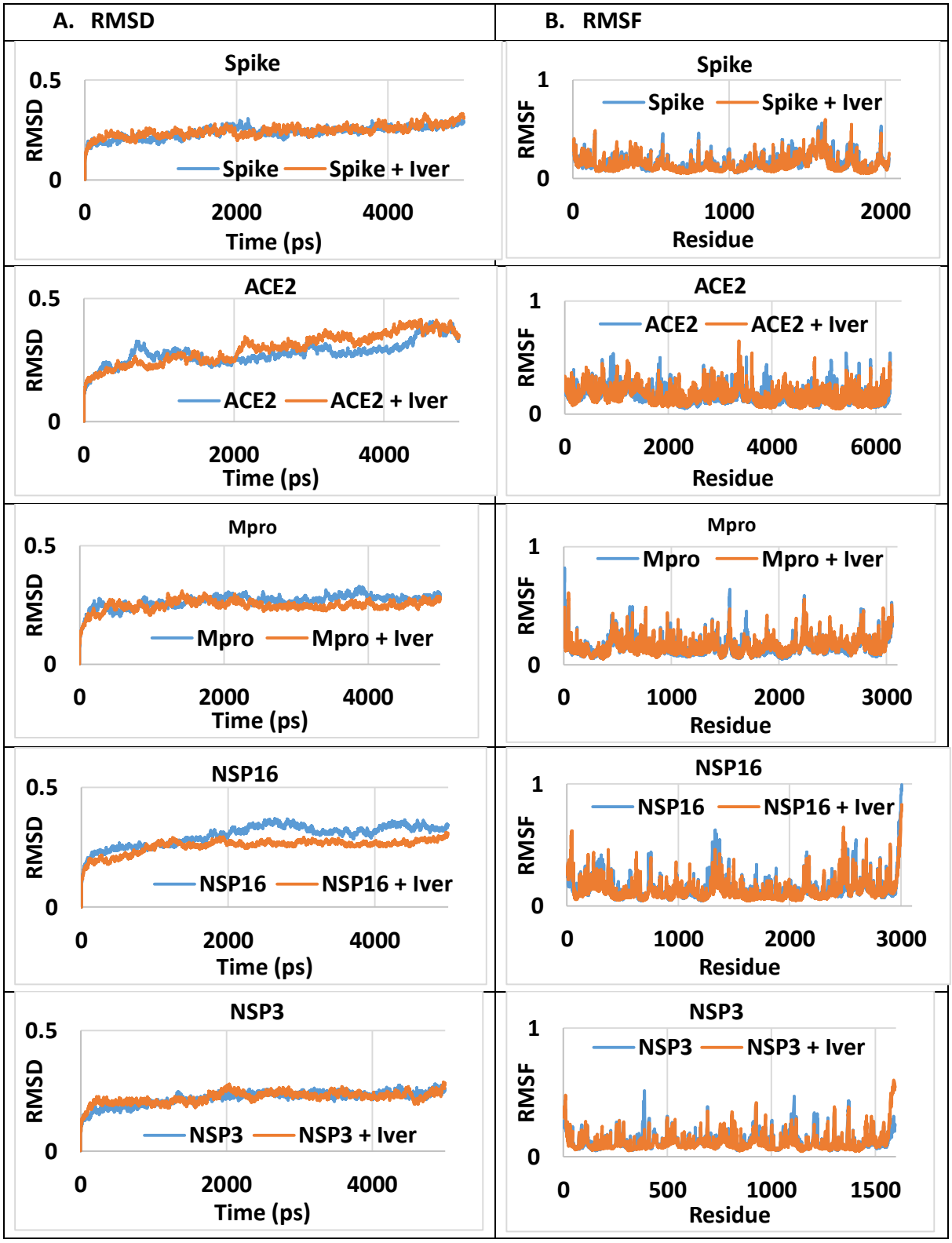
for viral infection. Any disturbance in the dimerization of NSP9 can be a way to overcome CoVs infection (Egloff et al. 2004; Hu et al. 2017). Whereas the function of NSP15 has a poorly defined but it was observed that a mutations in the active site of NSP15 abolish its endoribonuclease activity *in vitro* and reduces viral infectivity (Posthuma et al. 2006; Kang et

al. 2007). At the same time, any mutations outside of the active site are reported to have a larger effect on virus viability, indicating that NSP15 has role in CoVs infection apart from its function as an endoribonuclease (Ivanov et al. 2004; Kang et al. 2007; Bhardwaj, Liu, Leibowitz & Kao 2012). In our study, predicted binding energies of Ivermectin and doxycycline for N-protein are -8.2 and -6.3 kcal/mol respectively. The predicted binding energies of Ivermectin and doxycycline are -7.9 and -6.1 kcal/mol for NSP9 whereas -8.4 and -7.5 kcal/mol for NSP15. These finding show that Ivermectin have better binding affinity with N-protein, NSP9, and NSP15 compared to doxycycline. Altogether binding of Ivermectin with these proteins will hamper their individual function and decreases the viral genome replication, processing of virion packaging and ultimately viral load.

Molecular dynamics simulation: MD simulation is helpful in studying the stability of binding pose gained from docking, to rescore the inclusive binding energy as well as energy contribution by different residues, to study the conformational variations happening in protein due to ligand binding/unbinding etc. Thus, MD simulation was performed to examine the stability and dynamics of protein to confirm the docking results and get more insight into the stability of ligand-protein complex. MD simulations were carried out for SARS-CoV-2 spike, Mpro, NSP3, NSP16 and human ACE2 receptor and Ivermectin complex in the solvated states for 5000 ps. The results of MD simulations have been examined on the basis of root mean square deviation (RMSD) and root mean square fluctuation (RMSF). Based on the obtained MD trajectories, RMSD values of the protein backbone in complexes with the Ivermectin was computed. In all the cases, the RMSD values steadily increased from 0 to 200 ps, and reached equilibrium after that throughout the simulation period. RMSD was plotted with respect to time and showed stable behaviour throughout the simulation (Figure 6A). To explore the local protein flexibility, the time average of RMSF values of spike, Mpro, NSP3, NSP16 and ACE2 protein in presence Ivermectin over simulation period were calculated. RMSF with respect to amino acid was plotted and found no major fluctuation except few places where moderate fluctuation was observed (Figure 6B).

Most of the CoVs are known to bind with host metalloproteases (MMPs), predominantly for viral survival. Tetracyclines are known to chelate Zinc from MMPs (Aggarwal et al. 2010). Doxycycline being a tetracycline derivative, it will be beneficial in inhibition of SARS-CoV-2 infection because it chelates Zinc (Phillips, Gallagher & Weiss 2017). Tetracyclines also possess anti-viral activities and known to modulate innate immunity. Anti-viral activity of doxycycline had already been reported against retroviruses and dengue virus (Sturtz 1998; Yang, Chen, Tu, Yen & Yang 2007). They can decrease the expression of NF-kB and the release of inflammatory cytokines such as TNF-α, IL-1β and IL-6, independent of their antibiotic mechanism (Henehan, Montuno & De Benedetto 2017). Thus, they could participate in limiting the ‘cytokine storm’ induced in COVID-19 patients. The lipophilic nature of tetracyclines and their strong pulmonary penetration could allow them to inhibit viral replication at cellular level.

Figure 6. *Molecular dynamics simulation of Ivermectin complexed with Spike, ACE2, Mpro NSP16 and NSP3 proteins.*



Among tetracyclines, doxycycline has unique advantages such as long history of safety, the short duration of treatment and low cost. At the same time Ivermectin possess anti-viral activity for a range of the viruses. Very recently, it was observed that Ivermectin significantly inhibited SARS-CoV-2 under in vitro conditions (Caly et al. 2020).

In summary, the miraculous effect of combination of Ivermectin and doxycycline in COVID-19 patients is possibly by inhibition of spike-ACE2 interaction and inhibiting RNA dependent RNA polymerase, ADP Ribose Phosphatase, Endoribonuclease and NSP10-NSP16 complex mediated methyltransferase activities, anti-viral activity and chelation of the zinc & immunomodulatory property. Thus, the usage of Ivermectin and doxycycline combination will be an ideal choice in prevention and management of COVID-19.

Acknowledgement

The author would like to thank Dr S. Santosh Kumar, Radiation Biology & Health Sciences Division, Bhabha Atomic Research Centre, Mumbai for his valuable constructive suggestions and critical review of the article.

Declaration of Conflicting Interests

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ivermectin+Doxycycline MS.pdf (1.88 MiB) view on ChemRxiv • download file

Attachment 1: Product information for AusPAR Stromectol Ivermectin Merck Sharp Dohme (Australia) Pty Ltd PM-2012-01113-3-2 Final 30 October 2013. This Product Information was approved at the time this AusPAR was published.

IPC-MK0933-T-062010

PRODUCT INFORMATION

STROMECTOL® TABLETS

(ivermectin 3 mg)

NAME OF THE MEDICINE

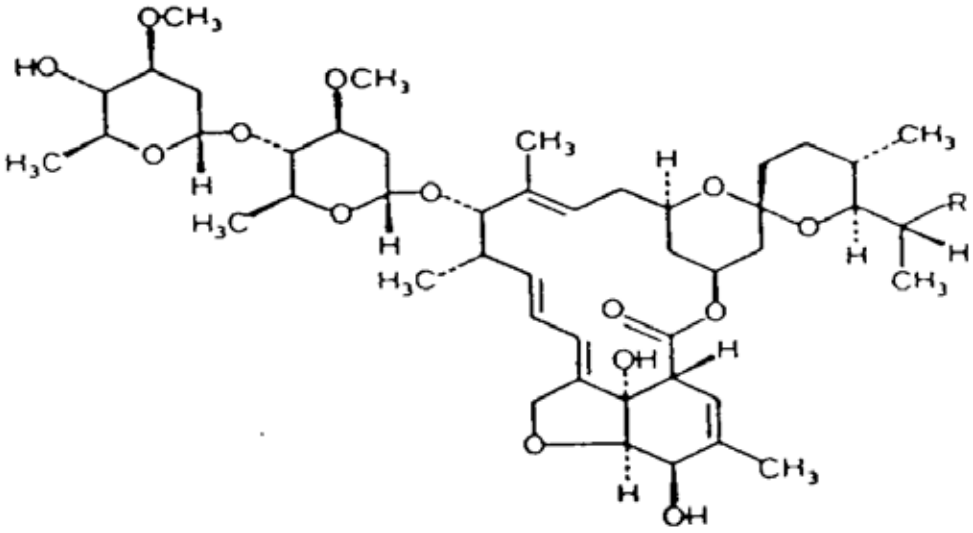
STROMECTOL® (ivermectin) 3 mg tablet

DESCRIPTION

Ivermectin is derived from the avermectins, a class of highly active broad-spectrum antiparasitic agents isolated from fermentation broths of *Streptomyces avermitilis*.

Ivermectin is a white to yellowish-white non-hygroscopic crystalline powder which is practically insoluble in water, freely soluble in methanol, and soluble in 95% ethanol.

The structural and molecular formulae follow.



Molecular Formulae:		Molecular Weight:
22, 23-dihydroavermectin B_{1a}	$C_{48}H_{74}O_{14}$ (R=C_2H_5)	875.10
22, 23-dihydroavermectin B_{1b}	$C_{47}H_{72}O_{14}$ (R=CH_3)	861.07

Ivermectin contains a minimum of 90% of 22, 23-dihydroavermectin B_{1a} (where the R group is ethyl) and a maximum of 10% of 22, 23-dihydroavermectin B_{1b} (the R group is methyl).

STROMECTOL is supplied as a 3 mg tablet. STROMECTOL tablets also contain microcrystalline cellulose, pregelatinised maize starch, magnesium stearate, butylated hydroxyanisole and citric acid anhydrous.

PHARMACOLOGY

Ivermectin inhibits signal transmission from the ventral cord interneurons to the excitatory motor neurones in nematodes by stimulating release of the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) from pre-synaptic nerve terminals. In arthropods, a similar mechanism inhibits signal transmission at the neuromuscular junction. Ivermectin does not readily penetrate the CNS of mammals, and thus does not interfere with mammalian GABA-dependent neurotransmission.

Pharmacokinetics:

Absorption and Distribution:

Ivermectin is incompletely absorbed (~50% bioavailable relative to an oral hydroalcoholic solution) following oral doses of ivermectin tablets, with a Tmax of ~4 hours. With 12 mg single dose tablets administered in healthy male volunteers, the mean peak plasma concentration of the major component was 46.6 (± 21.9) ng/mL (range 16.4-101.1 ng/mL).

Administration of 30 mg (333 to 600 µg/kg) ivermectin following a high-fat meal resulted in an approximate 2.5-fold increase in bioavailability relative to administration of 30 mg ivermectin in the fasted state in healthy volunteers.

Metabolism and Excretion:

Ivermectin is metabolised in humans, and ivermectin and/or its metabolites are excreted almost exclusively in the faeces over an estimated 12 days with less than 1% of the administered dose being excreted in the urine. The plasma half-life of ivermectin in man is about 12 hours (9.8-14.3 h) and that of the metabolites is about 3 days.

The pharmacokinetics of ivermectin has not been studied in patients with impaired hepatic or renal function.

CLINICAL TRIALS

Onchocerciasis in Adults:

A total of 758 adult patients with onchocerciasis received a single oral dose of 150-220 mg/kg ivermectin in three studies. In these studies, a significant reduction in microfilarial counts was reported in evaluable patients who received ivermectin, compared to those who received diethylcarbamazine or placebo. At 2-4 days after ivermectin dosing, microfilariae (mf) counts less than 5 per mg skin were reported in 29% to 96% of evaluable subjects, at 3 months after dosing < 5 mf/mg were reported in 88% to 96% of evaluable subjects, and at 12 months after dosing < 5 mf/mg were reported in 48% to 62% of evaluable patients. In patients with ocular involvement there was a significant reduction in intraocular microfilariae maintained for up to 12 months.

Onchocerciasis in Children:

In an open study, ivermectin reduced skin microfilaria in children with onchocerciasis for up to 12 months. One hundred and three (103) children aged between 5 to 12 years were treated with the targeted dose of 150 mm/kg ivermectin as a single oral dose. Resulting geometric mean microfilariae (mf) counts fell from a pre-treatment value of 36.4 mf/mg skin to 5.8%, 0.7%, 2.2% and 5.4% of the pre-treatment value on day 3, month 3, month 6 and month 12 respectively.

Intestinal Strongyloidiasis:

Two controlled clinical studies using albendazole as the comparative agent were carried out in countries where albendazole is approved for the treatment of strongyloidiasis of the gastrointestinal tract, and two controlled studies were carried out using thiabendazole as the comparative agent. Efficacy, as measured by cure rate, was defined as the absence of larvae in follow-up stool examinations post-therapy. Based on this criterion, efficacy was significantly greater for ivermectin (a single dose of 170 to 200 mg/kg) than for albendazole (200 mg b.i.d. for 3 days). Ivermectin administered as a single dose of 200 mg/kg for 1 day was as efficacious as thiabendazole administered at 25 mg/kg b.i.d. for 3 days.

Summary of Cure Rates for Ivermectin versus Comparative Agents in the Treatment of Strongyloidiasis

	Cure Rate* (%)	
	Ivermectin**	Comparative Agent
Albendazole***		
Comparative		
International Study	22/28 (79)	10/23 (43)
WHO Study	126/152 (83)	67/149 (45)
Thiabendazole†		
Comparative		
International Study	10/15 (67)	13/15 (87)
US Study	14/14 (100)	16/17 (94)

* Number and % of evaluable patients
** 170-200 mg/kg
*** 200 mg b.i.d. for 3 days
† 25 mg/kg b.i.d. for 3 days

Sarcoptes Scabiei:

Classic variety/Typical Scabies:

The clinical data evaluated for this indication were from a literature-based submission which uncovered 8 randomized controlled trials of ivermectin in typical scabies (see References), as well as a number of reviews, including a 2010 Cochrane Review (Strong and Johnstone, 2010).

Placebo-controlled studies

Macotela-Ruiz *et al*. (1993) conducted a trial comparing a single dose of ivermectin 200 µg/kg body weight with placebo in the treatment of scabies in 55 patients. The authors found that 26 (79.3%) patients were cured, defined as absence of itching and no dermatologically active lesions, with ivermectin on their first follow-up visit vs. 4 (16%) in the placebo group ($p<0.001$). The overall cure rates were 37 (74%) and 4 (16%) for ivermectin and placebo, respectively ($p<0.001$).

Active-comparator trials

Bachewar *et al*. (2009) conducted a non-blinded, prospective, randomized trial comparing benzyl benzoate 25% lotion (applied nightly for 2 nights), permethrin 5% cream (applied once), and a single dose of ivermectin 200 µg/kg body weight in 103 patients with noncrusted scabies. The primary outcome of interest was cure rate, defined as no lesions, measured after 1 and 2 weeks.

After 1 week, the cure rates were 76%, 82.1%, and 55.6% for benzyl benzoate, permethrin, and ivermectin, respectively ($p<0.05$ for permethrin vs. ivermectin). After 2 weeks, the cure rates were 92% for benzyl benzoate, 96.4% for permethrin, and 100% for ivermectin.

Chouela *et al*. (1999) compared ivermectin and lindane in a randomized, controlled, double-blind study in 53 patients with scabies. Patients received either a single oral dose of ivermectin 150-200 µg/kg body weight or topical application of 1% lindane solution. Treatment was repeated if clinical cure had not occurred, with clinical cure defined as absence of both pruritus and clinical lesions or a reduction in severity of signs and symptoms to 'mild'.

At day 15, 14/19 (74%) patients in the ivermectin group showed healing compared with 13/24 (54%) in the lindane group ($p=0.22$). At day 29, 18/19 (95%) patients receiving ivermectin were healed, and 23/24 (96%) receiving lindane were healed ($p>0.99$).Further analysis of the equivalence of therapeutic efficacy showed that ivermectin was statistically no less efficacious than lindane ($p<0.02$).

Ly *et al*. (2009) compared 2 regimens of benzyl benzoate (BB) and ivermectin in a randomized, open-label trial in 181 patients with scabies. BB was administered as either one application of 12.5% benzyl benzoate which was not removed for 24 hours (BB1), or as two such applications, each over 24 hours (BB2). Ivermectin was administered as a single oral dose of 150-200 µg/kg body weight.

The primary outcome measure was complete disappearance of visible lesions and itching at day 14. Patients who had clearly worsened at day 7 were given the same treatment as the week before. If no change was noted at day 7 or if the patient had improved, nothing further was done until day 14. If treatment failure was observed at day 14, the treatment was applied again. If treatment failure was observed again at day 28, patients in the BB1 and ivermectin groups were switched to the BB2 application, and those in the BB2 group were switched to ivermectin.

For the primary endpoint of efficacy at day 14, cure rates were 37/68 (54.4%) with BB1, 33/48 (68.8%) with BB2, and 16/65 (24.6%) with ivermectin, with a significance level of $p<10^{-6}$ overall. The comparison of BB1 and BB2 combined was also superior to ivermectin ($p<10^{-5}$). At day 28, cure rates were 52/68 (76.5%) for BB1, 46/48 (95.8%) for BB2, and 28/65 (43.1%) for ivermectin ($p<10^{-5}$ overall).

Madan *et al*. (2001) compared ivermectin and 1% topical lindane solution in a randomized trial in 200 patients with scabies. Patients received either a single dose of ivermectin 200 µg/kg body weight or 1% lindane topical solution applied overnight for 1 night. Efficacy was evaluated over the first 3 days, and at 2 weeks and 4 weeks. Improvement was measured on a categorical scale of: Complete/100% (no signs or symptoms of scabies); Good/75% (itching absent, lesions except burrows present; Moderate/50% (Itching markedly reduced, burrows +); Slight/25% (Itching slightly reduced, burrows ++); Poor/0% (no improvement); Negative (Further aggravation/exacerbation).

After 48 hours of treatment, no patients in either treatment group reported complete improvement, but 17.5% of patients in the ivermectin group and 7.1% in the lindane group reported good improvement. After 2 weeks, 36.1% of patients in the ivermectin group had complete improvement compared with 22.4% in the lindane group. At 4 weeks, 82.6% of patients in the ivermectin group had complete improvement compared with 44.4% in the lindane group. After 4 weeks, 2.8% of patients in the ivermectin group reported negative improvement compared with 4.9% in the lindane group.

Mushtaq *et al*. (2010) compared ivermectin and permethrin 5% cream in 100 patients with scabies. Patients received either a single dose of ivermectin 200 µg/kg body weight or a single application of topical permethrin 5% cream at night on the whole body for 12 hours. Patients not responding after the 1 week dose were given a second dose. Efficacy was determined by disappearance of itching, clearance of skin lesions, and absence of mites on microscopy of skin lesions.

Two weeks after the first dose, 24 (54.5%) patients in the ivermectin group were cured of disease, and 20 (47.6%) were cured in the permethrin group (p=0.5). Cure rates at 4 weeks were 79.5% and 88.1%, respectively (p=0.157). Itching at 4 weeks was mild in 6.8% of patients in the ivermectin group, and 2.4% in the permethrin group. No patients in either group had positive mite scrapings at week 2.

Nnoruka and Ague (2001) evaluated ivermectin and 25% benzyl benzoate topical application in an uncontrolled prospective study in 58 patients with scabies. Patients received ivermectin as a single oral dose at 200 µg/kg body weight or 25% benzyl benzoate applied from neck to toes for 72 hours. Clinical scores were calculated using skin lesions located in 16 different locations of the body. An area was given a score of 2 if there was at least one active lesion, a score of 1 if the most important lesion was healed, and a score of 0 if there was no lesion present. A classical burrow was scored a 2. A patient's clinical score was the sum off all individual area scores, which could range from 2 to 30 at study entry and from 0 to 30 at subsequent visits. In addition, pruritus was measured on a 10 cm visual analogue scale, where 0 = not present and 10 = extremely severe.

Mean clinical scores at baseline were 16.1 for ivermectin and 16 for benzyl benzoate. At day 7, scores were 5.4 and 10.3, respectively. At day 14 scores were 2.3 and 6.1, respectively, and at day 30 scores were 1.1 and 3.5 respectively. Analysis of variance showed a significant decrease in scores with ivermectin (p=0.004). At day 7, 31% of patients receiving ivermectin had complete disappearance of lesions and pruritus. On day 14, 65.3% of patients receiving ivermectin and 34.5% of patients receiving benzyl benzoate were healed. By day 30, 93.7% of patients receiving ivermectin were healed. By day 30, response to pruritus was rated as excellent in 93.1% of patients receiving ivermectin, and 48.3% in patients receiving benzyl benzoate.

Usha and Nair (2000) conducted a randomized trial comparing ivermectin and permethrin cream in the treatment of scabies in 85 patients and their family contacts. Ivermectin was administered as a single oral dose of 200 µg/kg body weight, and 5% permethrin cream was given as a single overnight topical application. Treatment was considered to be effective if, after 2 weeks, there was improvement in pruritus (measured on a visual analogue scale), clinical improvement in skin lesions with no new lesions, and absence of mites or the products on microscopy. Improvement was considered mild if there was less than 50% reduction in the number of lesions and pruritus; moderate if there was more than 50% reduction; and good if there was complete clearance. Treatment was considered a failure if, after 2 weeks, there was no improvement in pruritus and skin lesions, appearance of new lesions, or presence of mites or their products on microscopy. In the case of treatment failure, another dose of the randomized treatment was repeated. If the repeat treatment failed, patients were crossed over to the other treatment group. The primary endpoint was the complete disappearance of clinical signs and symptoms and no appearance of new lesions at the end of 2 months after the last dose was taken.

After the first week, 50% of patients in the ivermectin group and 84.3% of patients in the permethrin group had symptom improvement. By the second week, 70% in the ivermectin group and 97.8%, in the permethrin group improved. By the fourth week, 95% of patients in the ivermectin and 100% of patients in the permethrin groups improved. Two (5%) patients did not respond to 2 doses of ivermectin; both were cured after crossing over to permethrin after a single application. There was no recurrence of scabies at the end of 2 months of follow-up in either group. Complete clearance of lesions (graded as “good” improvement) occurred earlier with permethrin than with ivermectin, with significant differences at weeks 1 (p=0.03), 2 (p=0.003), and 4 (p=0.0005). At 8 weeks, all patients were completely cured (p=0.21).

Crusted variety:

No randomised controlled trials in crusted scabies have been reported in the literature. A systematic review of the literature on crusted scabies identified observational studies, case series and individual case reports in which 260 patients (mean age (range) 41.7 years (2-94 years)) received ivermectin. Of these cases, 182 received ivermectin at a dose of 200 µg/kg of body weight.

Seventy eight percent (78%) of all patients were managed in a clinic setting following confirmation of high mite count. The majority of patients for whom this was reported (70%) received ivermectin after proving refractory to classical topical treatments.

At least 72% of cases were treated with combination ivermectin and topical scabicide. In the large Australian cohort studies, all patients were administered topical keratolytic therapy in keeping with Australian clinical protocols.

Ivermectin was shown to have an overall clinical efficacy across these publications of an 87% cure rate, however, this must be interpreted with caution because of the potential for publication bias (i.e. publication of cases with good outcomes). The majority of patients presenting with mild to severe forms of crusted scabies were adequately managed with one to two oral doses of 200 µg/kg ivermectin. One to two doses may not be adequate in treating very severe cases of crusted scabies.

INDICATIONS

STROMECTOL (ivermectin) is indicated for the treatment of:

a) onchocerciasis and intestinal strongyloidiasis (anguillulosis).
b) crusted scabies in conjunction with topical therapy.
c) human sarcoptic scabies when prior topical treatment has failed or is contraindicated.

Treatment is only justified when the diagnosis of scabies has been established clinically and/or by parasitological examination. Without formal diagnosis, treatment is not justified in case of pruritus alone.

CONTRAINDICATIONS

Hypersensitivity to any component of the product.

PRECAUTIONS

If any hypersensitivity reaction to this product occurs, no further dose should be given.

Effects on fertility:

No data are available.

For treatment of onchocerciasis:

Ivermectin should be used only when infections with *O. volvulus* have been diagnosed or are suspected. No data are available to support its use prophylactically.

After treatment with microfilaricidal drugs, patients with hyperreactive onchodermatitis (sowda) may be more likely than others to experience severe adverse reactions, especially oedema and aggravation of onchodermatitis.

Rarely, patients with onchocerciasis who are also heavily infected with *Loa Loa* may develop a serious or even fatal encephalopathy either spontaneously or following treatment with an effective microfilaricide. In these patients, the following adverse experiences have also been reported: pain (including neck and back pain), red eye, conjunctival haemorrhage, dyspnoea, urinary and/or faecal incontinence, difficulty in standing/walking, mental status changes, confusion, lethargy, stupor, seizures or coma. This syndrome has been seen very rarely following the use of ivermectin.

Impaired Renal or Hepatic function:

Ivermectin has not been studied in patients with impaired hepatic function or impaired renal function. As ivermectin is extensively metabolised by the liver, caution should be exercised if ivermectin is administered to patients with impaired hepatic function.

Use in Pregnancy (Category B3):

Ivermectin should not be used in pregnancy as safety in pregnancy has not been established. Ivermectin caused cleft palates in mice and rats at oral doses of 0.4 and 10 mg/kg/day respectively, and cleft palates and clubbed feet in rabbits dosed at 3 mg/kg/day.

Use in Lactation:

Ivermectin is excreted in breast milk and safety in newborn infants has not been established. In rats, reduced survival occurred in control pups and pups exposed in utero that were cross-fostered to treated dams, but not in control pups or pups exposed in utero cross-fostered to control dams.

The drug should be given to nursing mothers only if the benefit to the mother outweighs the potential risk to the breast-fed infant, and the treatment of mothers who intend to breast-feed their infants should be delayed until at least one week after the birth of the child.

Carcinogenicity/Mutagenicity:

There have been no carcinogenicity studies with ivermectin. Ninety-four and 105 week carcinogenicity studies on mice and rats respectively were conducted with the closely related compound abamectin and were negative at up to 8 mg/kg/day in mice and up to 2 mg/kg/day in rats. Ivermectin was negative in three *in vitro* assays for geno toxicity (mutagen assays in bacteria and mouse cells, and unscheduled DNA synthesis in human cells). No tests have been done to test the potential of ivermectin for producing clastogenicity.

Paediatric Use:

Onchocerciasis:

Ivermectin should not be used in children under five years of age as safety in this age group has not been established.

The safety profile of ivermectin in children 5 to 12 years of age is similar to that observed in adults (see ADVERSE EFFECTS/Onchocerciasis).

Strongyloidiasis:

Efficacy has not been established in children under twelve years of age.

***Sarcoptes scabiei* (scabies):**

Ivermectin should not be used in children under 15 kg and under 5 years of age as safety in these groups has not been established.

Use in the Elderly:

Clinical studies of STROMECTOL did not include sufficient numbers of elderly subjects aged 65 years and over to determine whether they respond differently from younger subjects. In general, treatment of elderly patients should be cautious, reflecting the greater frequency of decreased hepatic, renal or cardiac function, and of concomitant disease or other drug therapy.

Effects on laboratory tests:

No data are available.

INTERACTIONS WITH OTHER MEDICINES

Interactions between ivermectin and other drugs have not been studied in clinical trials.

Very rare post-marketing reports of increased INR (International Normalised Ratio) have been reported when ivermectin was co-administered with warfarin.

ADVERSE EFFECTS
A. CLINICAL TRIAL AND PUBLISHED DATA

A.1. Strongyloidiasis:

Ivermectin has been demonstrated to be generally well tolerated in the treatment of strongyloidiasis.

In three clinical studies involving a total of 109 patients given either one or two doses of 170-200 mg/kg of ivermectin, the following adverse reactions were reported as possibly, probably, or definitely related to ivermectin:

Body as a whole: asthenia/fatigue (0.9%), abdominal pain (0.9%)

Gastrointestinal: anorexia (0.9%), constipation (0.9%), diarrhoea (1.8%), nausea (1.8%), vomiting (0.9%)

Nervous System/Psychiatric: dizziness (2.8%), somnolence (0.9%), vertigo (0.9%), tremor (0.9%)

Skin: pruritus (2.8%), rash (0.9%), and urticaria (0.9%)

Ivermectin was better tolerated than thiabendazole in comparative studies involving 37 patients treated with thiabendazole.

In a WHO sponsored study, children and adults (9-22 years) with strongyloidiasis were treated with ivermectin 200 mg/kg as a single dose or albendazole 200 mg twice daily for 3 days. The safety results are summarised in the following table:

WHO Study - Number of Patients Developing Adverse Effects Within 3 Days After Treatment, Regardless of Drug Relationship

Body System/ Symptoms	Ivermectin (n=163)	Albendazole (n=170)
Body As A Whole		
Abdominal Distension	7*	1
Chest Pain/Tightness	7*	0
Fever	10	7
Digestive System		
Loose Stools	16	17
Nausea	5	6
Watery Diarrhoea	2	3
Nervous System		
Headache	15	18
Dizziness, vertigo	5	10
Skin		
Diffuse Itching	3	6
Respiratory System		
Cough	11	8

* = $p < 0.05$

The Mazzotti-type and ophthalmologic reactions associated with the treatment of onchocerciasis or the disease itself would not be expected to occur in strongyloidiasis patients treated with ivermectin (See ADVERSE EFFECTS, Onchocerciasis).

Laboratory Test Findings:

In clinical trials involving 109 patients given either one or two doses of 170-200 mg/kg ivermectin, the following laboratory abnormalities were seen irrespective of drug relationship: elevation in ALT and/or AST (2%), decrease in leukocyte count (3%). Leukopenia and anaemia were seen in one patient.

A.2. Onchocerciasis:

Ivermectin has been demonstrated to be generally well tolerated in the treatment of onchocerciasis.

Historical data have shown that microfilaricidal drugs, such as diethylcarbamazine citrate (DEC-C), might cause cutaneous and/or systemic reactions of varying severity (the Mazzotti-type reaction) and ophthalmological reactions in patients with onchocerciasis. These reactions are probably due to allergic and inflammatory responses to the death of microfilariae. Patients treated with ivermectin for onchocerciasis may experience these reactions in addition to clinical adverse reactions possibly, probably, or definitely related to the drug itself.

In clinical trials involving 963 adult patients treated with 100 to 200 mg/kg ivermectin and 315 patients on placebo, worsening of the following Mazzotti-type reactions during the first 4 days post-treatment were reported (ivermectin, placebo, respectively): arthralgia/synovitis (9.3%, 4.4%), axillary lymph node enlargement (11.0%, 2.9%), axillary lymph node tenderness (4.4%, 1.0%), cervical lymph node enlargement (5.3%, 4.1%), cervical lymph node tenderness (1.2%, 0.6%), inguinal lymph node enlargement (12.6%, 6.7%), inguinal lymph node tenderness (13.9%, 5.7%), other lymph node enlargement (3.0%, 1.6%), other lymph node tenderness (1.9%, 0.6%), pruritus (27.5%, 17.2%), skin involvement including oedema, papular and pustular or frank urticarial rash (22.7%, 9.2%), and fever (22.6%, 4.8%).

In clinical trials, ophthalmological conditions were examined in 963 adult patients before treatment, at day 3 and months 3 and 6 after treatment with 100 to 200 mg/kg ivermectin. Changes observed were primarily deterioration from baseline 3 days post-treatment. Most changes either returned to baseline condition or improved over baseline severity at the month 3 and 6 visits. The percentages of patients with worsening of the following conditions at day 3, month 3 and 6, respectively, were: limbitis: 5.5%, 4.8%, and 3.5% and punctate opacity: 1.8%, 1.8%, and 1.4%. The corresponding percentages for patients treated with placebo were: limbitis: 6.2%, 9.9% and 9.4% and punctate opacity: 2.0%, 6.4% and 7.2%.

In clinical trials involving 963 adult patients who received 100 to 200 mg/kg ivermectin and 315 patients on placebo, the following clinical adverse reactions were reported as possibly, probably, or definitely related to the drug in ³ 1% of the patients (ivermectin and placebo, respectively): facial oedema (1.2%, 0%), peripheral oedema (3.2%, 0.6%), orthostatic hypotension (1.1%, 0%), and tachycardia (3.5%, 0.6%). Drug-related headache and myalgia occurred in < 1% of patients given ivermectin (0.2%, and 0.4%, respectively). However, these were the most common adverse experiences reported overall during these trials regardless of causality (22.3%, and 19.7%, respectively).

A similar safety profile was observed in an open study in paediatric patients ages 5 to 12.

The following ophthalmological side effects do occur due to the disease itself but have also been reported after treatment with ivermectin: abnormal sensation in the eyes, eyelid oedema, anterior uveitis, conjunctivitis, limbitis, keratitis, and chorioretinitis or choroiditis. These have rarely been severe or associated with loss of vision and have generally resolved without corticosteroid treatment.

Laboratory Test Findings:

In controlled clinical trials, the following laboratory adverse experiences were reported as possibly, probably, or definitely related to the drug in ³ 1% of the patients (ivermectin and placebo, respectively): oeosinophilia (3%, 0%) and haemoglobin increase (1%, 0%).

A.3. *Sarcoptes scabiei* (scabies):

Adverse effects reported in the literature review were similar to those reported in clinical trials for the other indications. The most common adverse drug reactions reported with ivermectin in the review involved the transient exacerbation of pruritus that can sometimes occur as a result of sensitisation of the human host to mite antigens, with a consequent immunologic reaction (1.4%). Sensitisation also frequently results in delayed resolution of symptoms. Patients should be warned that itching may persist for one to two weeks after treatment, even if the mite is successfully eradicated. Other frequently reported reactions consisted of headache (< 1.0%), arthralgia (< 1.0%) and anorexia (< 1.0%). Additionally, lethargy (< 1.0%), listlessness (< 1.0%), abdominal discomfort (< 1.0%), rash (< 1.0%), and dizziness (< 1.0%) were reported. Up to 1.86% of patients noticed that they had passed Ascaris worms during the week after treatment.

B. POST-MARKETING EXPERIENCE

Onchocerciasis:

Conjunctival haemorrhage

All Indications:

Very rarely, hypotension (mainly orthostatic hypotension), worsening of bronchial asthma, toxic epidermal necrolysis, Stevens-Johnson syndrome, seizures, elevation of liver enzymes and elevation of bilirubin.

DOSAGE AND ADMINISTRATION

STROMECTOL is available as white tablets each containing 3 mg ivermectin. Treatment is administered as a single oral dose given with water.

The dose is determined by the patient's weight as shown below.

Strongyloidiasis - the dosage aims to provide approximately 200 µg ivermectin/kg body weight.

Dosage in Strongyloidiasis:	
Body Weight (Kg):	Dose (Number of 3 mg tablets):
15-24	One
25-35	Two
36-50	Three
51-65	Four
66-79	Five
≥ 80	Approx 200 mg/kg
In general, additional doses are not necessary, however, a follow-up examination of stool to verify efficacy should be performed.	

Onchocerciasis - the dosage aims to provide approximately 150 µg ivermectin/kg body weight.

Dosage in Onchocerciasis:	
Body Weight (Kg):	Dose (Number of 3 mg tablets):
15-25	One
26-44	Two
45-64	Three
65-84	Four
Alternatively, the dose of ivermectin for use in mass chemotherapy campaigns may be determined by the patients height, as follows:	
Height (cm):	Dose (Number of 3 mg tablets):
90-119	One
120-140	Two
141-158	Three
> 158	Four
[Reference: Alexander, N.D.E., ivermectin dose assessment without weighing scales. Bulletin of the World Health Organisation, 71 (3/4): 361-366 (1993)]	
The suggested dose interval for most patients is 12 months. At some sites it may be preferable to use a 6 months interval depending on such considerations as density of skin microfilariae and/or prevalence.	

***Sarcoptes scabiei* (scabies)** - the dosage aims to provide approximately 200 µg ivermectin/kg body weight per dose.

Dosage in Sarcoptes scabiei (scabies)	
Body Weight (Kg):	Dose (Number of 3 mg tablets):
15-24	One
25-35	Two
36-50	Three
51-65	Four
66-79	Five
> 80	Approx 200 mg/kg

Classic/typical scabies: 2 doses (1 dose on day 1 and another dose between day 8 and day 15). Ivermectin can be used alone or in combination with a topical scabicide.

Crusted scabies (ivermectin in combination with a topical scabicide administered as):

- Mild cases: 2 doses (1 dose on day 1 and another dose between day 8 and day 15)
- Moderate to severe cases: More than 3 doses may be required for effective treatment.

Patients with crusted scabies should use keratolytics on days they are not treated with topical scabicides to assist with the reduction of scaling that harbours the mite. Consultation with a dermatologist or infectious diseases physician is recommended.

The life cycle of *S. scabiei* begins with the pregnant female laying two to three eggs a day in burrows several millimetres to several centimetres in length in the stratum corneum (outermost layer) of the skin. The eggs hatch in two weeks. The larvae form intra-epidermal lesions whilst they mature into the adult form. This maturation takes 2-3 weeks.

OVERDOSAGE

There are reports of accidental overdosing of ivermectin, but no fatalities have been attributable to ivermectin overdosing.

In significant accidental intoxication with unknown quantities of a veterinary formulation, symptoms have resembled those seen in animal toxicology studies, which were chiefly rash, contact dermatitis, oedema, headache, dizziness, asthenia, nausea, vomiting, diarrhoea, mydriasis, somnolence, depressed motor activity, tremors and ataxia. Other adverse effects that have been reported include: seizure, dyspnoea, abdominal pain, paraesthesia and urticaria.

In case of accidental poisoning, supportive therapy, if indicated, should include parenteral fluids and electrolytes, respiratory support (oxygen and mechanical ventilation if necessary) and pressor agents if clinically significant hypotension is present. Induction of emesis and/or gastric lavage as soon as possible, followed by purgatives and other routine antipoison measures may be indicated if needed to prevent absorption of ingested material. Although data are not available for man, it would appear advisable to avoid GABA-agonistic drugs in the treatment of accidental ivermectin intoxication.

In a study in which healthy volunteers were orally administered up to 2000 µg/kg ivermectin in a fasted state or up to 600 µg/kg ivermectin following a high-fat (48.6 g of fat) meal, there were no indications of central nervous system toxicity observed at any dose irrespective of food intake.

For information on the management of overdose, contact the Poison Information Centre on 131126 (Australia).

PRESENTATION AND STORAGE CONDITIONS

Presentation:

STROMECTOL is available as white, round, flat tablets with a bevelled edge, engraved with MSD on one side and 32 on the other side and containing 3 mg ivermectin. The tablets are packaged in aluminium foil blister packs in cardboard cartons. Pack size: 4 tablets.

Storage Instructions:

Store below 30°C.

NAME & ADDRESS OF THE SPONSOR

Merck Sharp & Dohme (Australia) Pty Ltd
54-68 Ferndell Street
South Granville NSW 2142
Australia

POISION SCHEDULE OF THE MEDICINE

Prescription Only Medicine (S4)

Date of first inclusion in the Australian Register of Therapeutic Goods (the ARTG):
Approved by the Therapeutic Goods Administration: 27th August 1999.

Date of most recent amendment (scabies indication registration):
15 July 2013

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Am J Clin Nutr. Author manuscript; available in PMC 2008 April 21.

FOI 26-3132 - Document 5

Published in final edited form as:
Am J Clin Nutr. 2007 October ; 86(4): 1167–1173.

Serum zinc and pneumonia in nursing home elderly

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Abstract

Background—Zinc plays an important role in immune function. The association between serum zinc and pneumonia in the elderly has not been studied.

Objective—To determine if serum zinc concentrations in nursing home elderly are associated with incidence and duration of pneumonia, total and duration of antibiotic use, and pneumonia-associated and overall deaths.

Design—This observational study was conducted in residents from 33 nursing homes in Boston, MA, who participated in a one-year randomized, double-blind, and placebo controlled vitamin E supplementation trial; all were given daily doses of ½ RDA of essential vitamins and minerals including zinc. Participants with baseline (N=578) or final (N=420) serum zinc concentrations were categorized as having low (<70 μg/dL) or normal (≥70 μg/dL) serum zinc concentrations. Outcome measures included incidence and number of days with pneumonia, number of new antibiotic prescriptions, days of antibiotic use, death due to pneumonia, and, all-cause mortality.

Results—Subjects with normal final serum zinc concentrations had lower pneumonia incidence, total antibiotic use (by almost 50%), and shorter duration of pneumonia and antibiotic use (by 3.9 and 2.6 days, respectively) (all p-values ≤0.004) relative to those with low zinc concentrations. Normal baseline serum zinc concentrations were associated with decreased all-cause mortality (p=0.049).

Conclusion—Normal serum zinc concentrations in nursing home elderly were associated with decreased incidence and duration of pneumonia, and decreased use and duration of antimicrobial therapy. Zinc supplementation to maintain normal serum zinc concentrations in the elderly may help reduce pneumonia incidence and associated morbidity.

Keywords

serum zinc; nursing home elderly; pneumonia; mortality; antibiotic use

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INTRODUCTION

Pneumonia is a major public health problem in the elderly (1). An important predisposing factor to the increased incidence of infections such as pneumonia in the elderly is the age-associated decline in immune function (2). Such changes in immune response with age, in addition to malnutrition, contribute to the increased frequency and severity of pneumonia, as well as mortality due to pneumonia in the elderly (1-4).

Zinc has been shown to play an important role in regulation of the immune response particularly T cell-mediated function (5-7). Zinc deficiency has been shown to cause thymus involution and to depress lymphocyte proliferation, interleukin-2 (IL-2) production, delayed type hypersensitivity skin responses, and antibody response to T cell-dependent antigens (5,8). Similar defects in T cell function have been observed with aging (2). Several investigators have reported low zinc status or decreased intake in elderly subjects (9-11). Furthermore, low zinc status in the elderly has been shown to contribute to age-associated dysregulation of the immune response (3,12) and zinc supplementation has been shown to improve T cell mediated function in elderly (9,12-15) . In children, low concentrations of circulating zinc have been shown to be associated with an increased risk of respiratory morbidity (16), and zinc supplementation has been shown to reduce both the risk and duration of pneumonia and deaths due to pneumonia in children (17,18). The association between serum zinc and pneumonia in the elderly, however, has not been studied.

From April 1998 to August 2001, a randomized controlled trial was carried out in order to investigate the effect of vitamin E supplementation on respiratory infections in a nursing home elderly population (19). We found a high proportion (about 30%) of nursing home elderly with low serum zinc concentrations at baseline, and after a year of follow up despite all participants' receiving ½ RDA of essential vitamins and minerals, including zinc, during the trial. Because recently published studies in children (17,18) have shown zinc supplementation to be beneficial in reducing morbidity and mortality from pneumonia and past research has demonstrated the negative impact of zinc deficiency on immune function in the elderly, we examined the relationships between serum zinc concentration and the incidence and duration of pneumonia, total antibiotic use, and duration of antibiotic use and death due to pneumonia, as well as overall deaths in this population of elderly nursing home residents.

SUBJECTS AND METHODS

Study design and intervention

A total of 617 subjects was enrolled (approximately 1/3 enrolled in each of 3 successive years) into a randomized, double-blind, placebo-controlled trial of the effect of one-year vitamin E supplementation (200 IU/day) on respiratory infections in a nursing home population (19). The Tufts-New England Medical Center Institutional Review Board approved the study protocol and informed consent form.

Nursing home residents have a heterogeneous intake of micronutrients (20), some of which are necessary for proper immune function. To reduce variability in the vitamin E trial, all subjects received a daily capsule containing 50% of the Recommended Dietary Allowance (RDA) (21) for essential micronutrients, including zinc. Fifty percent RDA was selected since few subjects meeting our eligibility criteria would have dietary intakes <50% of the RDA for micronutrients (22).

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At baseline as well as at follow-up, about 30% of subjects were found to be deficient in zinc. Other micronutrient deficiencies were much less prevalent at baseline (from 0 to 10%) and their prevalence did not significantly change at follow up (19).

Selection of study subjects

Detailed information on the screening and recruitment of participants has been described previously (19). The eligibility criteria included age ≥65 y, life expectancy >6 months, no anticipated discharge within 3 months, not room-bound for the past 3 months, body mass index (BMI) ≥16 kg/m^2, serum albumin ≥3.0 g/dL, ability to swallow pills, and willingness to receive influenza vaccine and provide informed consent. Exclusion criteria included active neoplastic disease, tube feeding, kidney dialysis, intravenous or urethral catheters for the last 30 days, tracheostomy or chronic ventilator use, chronic steroid treatment >10 mg/d, use of immunosuppressive drugs, use of antibiotics within the prior 2 weeks, and >RDA level supplements of vitamins E, C, B_6, selenium, zinc, β-carotene, or fish oil.

The current analyses include all enrolled subjects with baseline (N=578) or end of study zinc measurements (N=420, including 7 subjects with only end of study zinc values). For the purposes of this study, subjects were categorized by serum zinc concentrations using cutoffs of <70 μg/dL to indicate low serum zinc concentrations and ≥70 μg/dL to indicate normal serum zinc concentrations (23).

Outcomes

Pneumonia-related outcomes included incidence of and number of days with pneumonia, new antibiotic prescriptions for pneumonia, days of antibiotic use for treatment of pneumonia, and death due to pneumonia. We also examined all-cause mortality.

Data collection

Information regarding subject characteristics, baseline diseases and medications, and vaccination history was obtained from medical records. Fasting blood was collected at baseline and at study completion for clinical chemistries, complete blood count, and nutritional status as previously described (19). Serum samples were collected using trace metal-free tubes and serum zinc concentrations were measured using the Perkin Elmer flame atomic absorption spectrometer (24) at the Nutrition Evaluation Laboratory, Human Nutrition Research Center on Aging at Tufts University.

Study nurses were trained by a study physician to identify relevant respiratory symptoms and to perform a focused physical examination of the respiratory tract. Supervised practice evaluations were repeated throughout the study to reinforce the nurses' clinical skills and to ensure consistency of the pneumonia data collection. The study nurses collected information weekly relating to infection, including symptoms of respiratory infections, temperature, respiratory and heart rate, and a physical examination focused on the respiratory system. The nurses reviewed each participant's chart for documentation of laboratory analyses, radiography, medications, micronutrient supplementation, weight, and nursing or physician descriptions of symptoms and signs relating to pneumonia and other respiratory infections. At the end of the study, data collected from the subjects were randomly assigned, by nursing home, to the two study physicians for diagnosis of infections. Infection data from any one subject were evaluated by only one physician, except for 18 subjects whose records were used to determine the concurrence of diagnoses between physicians.

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Diagnosis of pneumonia

Details on how the study physicians evaluated the data to determine incidence and duration of pneumonia have been described previously (19). Briefly, clinical definitions of pneumonia were developed based on accepted definitions (25). In order to increase the specificity of the definitions, a diagnosis of pneumonia had to include at least one physical sign and thus could not be made on symptoms alone. An episode was considered resolved when all symptoms ceased. A new infection was defined as one occurring after 7 symptom-free days. Pneumonia symptoms could include cough with or without sputum production, chest pain, dyspnea, and fever. Signs of infection included elevated temperature (≥38°C), tachycardia, tachypnea, abnormal breath sounds, and dullness to percussion of the chest. The diagnosis required radiological findings of 1 or more new pulmonary infiltrates.

Statistical analysis

The demographic and clinical characteristics of participants in the low and normal serum zinc groups were compared by using Student's t-test for independent samples (continuous measures) and Fisher's exact test (categorical measures).

As noted above, all subjects received a capsule containing 50% of the RDA for essential micronutrients, including zinc. Because of this supplementation, the baseline zinc concentrations might not reflect the usual zinc status during the entire study period. Therefore, we also used zinc concentrations measured at the final study visit as a marker of zinc status. However, analyses based on final zinc concentrations were necessarily limited to those who survived or completed the trial.

Rate ratios and their confidence intervals for incidence of pneumonia and number of antibiotic prescriptions per year were modeled using Poisson regression with the natural logarithm of time as an offset (26). None of the deviance statistics from the Poisson regressions exceeded 1, which suggests that there was no overdispersion. Multiple linear regression analyses were used to determine the association between serum zinc and duration of pneumonia and antibiotic use due to pneumonia. Cox-proportional hazards models using baseline zinc concentrations were fitted to determine the hazards ratio for deaths due to pneumonia or overall deaths using baseline zinc concentrations. Except for the proportional hazards analyses, these analyses were performed by using both baseline and final zinc concentrations as predictors. Although the original study did not show a significant difference in the rate of pneumonia between the vitamin E and placebo groups (19), we nevertheless controlled for allocation to treatment and placebo groups in these analyses. We also adjusted for year of enrollment, as well as age, sex, baseline BMI, current smoking, diabetes mellitus, and chronic obstructive pulmonary disease (including bronchial asthma), which have been shown to be associated with increased risk of pneumonia and/or pneumonia-associated death (1,27,28). Analyses done comparing those with low compared to normal final serum zinc concentrations were also controlled for change in BMI between baseline and follow-up values. Additionally, in separate analyses, we also controlled for baseline serum albumin and change in serum albumin concentrations between baseline and follow-up in these models. Two-sided observed significance concentrations (*p* values) <0.050 were considered to be statistically significant. All calculations were performed by using SAS for Windows, version 9.1.2 (SAS Institute Inc, Cary, NC, USA).

RESULTS

The demographic and clinical characteristics of participants with baseline zinc (N=578) and those with final study zinc concentrations (N=420) were similar (Table 1). These characteristics were also similar in distribution to those of our original overall study population (N=617) (19).

FOI 26-3132 - Document 5

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Of the characteristics listed in Table 1, only the difference in age between those with low and normal baseline serum zinc concentrations was statistically significant (mean ± SD were 86 ± 8 and 84 ± 7 years, respectively; $p<0.001$). Elderly with low final serum zinc concentrations were older at baseline (86 ± 8 versus 84 ± 7 years; $p=0.014$), and were more likely to have had coronary artery disease (39% versus 28%; $p=0.042$) at baseline compared to those with normal final zinc concentrations. All subjects received influenza vaccine and there was no difference between the two groups in the proportion of subjects who had pneumococcal immunization.

When participants with low and normal baseline zinc concentrations were compared, differences in the incidence and duration of pneumonia, and total amount and duration of antibiotic use due to pneumonia (Table 2) were not statistically significant. Deaths due to pneumonia were 53% lower in those with normal compared to low baseline serum concentrations of zinc, but this association was not statistically significant ($p=0.198$). All-cause mortality rate was 39% lower in those with normal versus lower baseline zinc concentrations ($p=0.049$) (Table 2).

End of study serum zinc concentrations were strongly associated with the incidence and duration of pneumonia as well as antibiotic use and duration of antimicrobial therapy (Table 3; $p\leq0.004$ in all cases). The incidence of pneumonia and the total number of antibiotics used for treatment of pneumonia were about 50% lower in those with end serum zinc concentrations ≥70 μg/dL compared to those with concentrations <70 μg/dL. In addition, the duration of pneumonia was lower by 3.9 days and the duration of antibiotic use by 2.6 days in those with end serum zinc in the normal range compared to those with low concentrations (Table 3). Controlling for coronary artery disease in our multiple regression analyses model did not change the statistical significance of the differences observed. In addition, differences remained statistically significant after controlling for baseline serum albumin and change in serum albumin concentrations between baseline and follow-up. The 310 subjects with normal end zinc concentrations at completion suffered 78 cases of pneumonia while the 110 with low end zinc concentrations suffered 51. The fraction of pneumonias occurring in the final 2 months of the study was similar in those subjects with normal (14 cases out of 78) and low (8 cases out of 51) end serum zinc concentrations, i.e., 18% vs 16%, respectively.

Our findings were specific to zinc. We did not observe similar associations in our outcome variables of interest with other micronutrients that might influence immune function such as vitamins A, B6, D, E, β-carotene, and iron (data not shown). In addition, the mean changes in weight and BMI from baseline to follow-up between subjects with low and normal zinc concentrations were not significantly different; mean weight changes were −0.80 and −0.01 kg, respectively, and BMI, −0.35 and −0.01 kg/m^2, respectively.

DISCUSSION

We observed that elderly nursing home residents with low serum zinc concentrations at the end of one year of 50% RDA micronutrient daily supplementation, including zinc, had a higher risk of pneumonia and longer duration of pneumonia episodes as well as increased antibiotic use and longer duration of antibiotic use for treatment of pneumonia episodes. In addition, low baseline concentrations of serum zinc in our elderly nursing home population were associated with increased all-cause mortality.

Our finding of a significantly lower all-cause mortality rate (by 39%) between those with normal versus low baseline serum zinc concentrations among elderly in this study suggest that zinc may play a crucial role in influencing mortality in the elderly. Severe zinc deficiency can impair immunity and increase susceptibility to infectious diseases, a major cause of mortality in the elderly (1,3,29,30). Indeed, the risk of mortality was reduced by 27% in participants of

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the Age-related Eye-Disease Study (aged 55 to 81 years) who received zinc supplements (RR: 0.73; 95% CI, 0.61−0.89) (31). In addition, zinc supplementation has been shown to reduce overall mortality by as much as 51% in children with diarrhea and 68% in infants born full-term and small for gestational age (32,33).

When baseline serum zinc status was used as a measure of zinc to determine if low serum zinc affects susceptibility to pneumonia, differences failed to reach statistical significance. It may be that findings using baseline zinc concentrations were attenuated due to the higher risk of death among subjects with low baseline zinc concentrations or due to loss of subjects from serious illnesses and/or hospitalizations. In addition, because all study participants were provided with ½ RDA supplement that included zinc, the baseline concentrations may not reflect zinc status during much of the study period.

Because of the potential bias introduced by zinc supplementation, we performed similar analyses using final zinc concentrations, as this measure may better reflect the zinc status of subjects during the course of the study. We observe a strong association between low final zinc concentrations with increased incidence and duration of pneumonia, as well as total and duration of antibiotic use for pneumonia treatment. As described in the results, the effects observed were specific to zinc and were not observed with other micronutrients. Furthermore, the lower incidence and morbidity of pneumonia observed in subjects with normal final zinc concentrations compared to those with low final zinc concentrations were not due to differences between the two groups in changes in weight, BMI, or other micronutrients (19) during the study period.

Zinc deficiency has been suggested to be a risk factor for immune deficiency and subsequent infection relapses in the elderly (12,29,34). Zinc is essential for membrane integrity, DNA synthesis and cell proliferation, in addition to being a cofactor to more than 300 enzymes (5, 35).

It is essential for all highly proliferating cells in the human body, especially the immune system (36). Thymus atrophy and lymphopenia, as well as other defects in T cell function have been observed in both zinc deficiency and in the elderly (2,5,8).

Low zinc consumption has been reported in the elderly (10,37). Bogden et al. reported that zinc ingestion was below the RDA in more than 90% of their 100 study subjects aged 60−89 years (37). In addition, as mentioned above, we found below normal serum zinc concentrations in approximately 30% of the nursing home elderly enrolled in our vitamin E supplementation study. This level of deficiency persisted during the study period despite giving the participants supplements with ½ RDA of zinc (19). Thus, our finding of a significant association between low serum zinc level and pneumonia could be of major importance to the health of elderly, as poor zinc status would lead to impaired immune response, which could in turn result in increased susceptibility to infections in the elderly (9,29,34). Supplementing with ½ RDA of zinc appeared to benefit many of the subjects with inadequate baseline zinc concentrations, but the results also showed that this supplementation was not adequate to maintain serum zinc concentrations in a significant proportion of subjects.

Plasma or serum zinc concentrations are also known to decrease sharply in many infections (35,38,39). The decline is part of a set of metabolic reactions to infection known as the acute phase response (40). Although one could argue that lower serum zinc concentrations might be due to a higher incidence of pneumonia, this is unlikely to be the case here as the acute effect of pneumonia on zinc is likely to be transient--approximately 2 weeks if effective therapy is provided (41). Given the similar incidence of pneumonia in our participants in the last months of the study, transient suppression of serum zinc by the acute phase response is unlikely to be responsible for the low end study zinc concentrations.

FOI 26-3132 - Document 5

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Zinc supplementation may play an important role in the prevention and/or modulation of infectious diseases in the elderly (3,9,14,29). Various studies on zinc supplementation in the elderly have observed increased circulating zinc concentrations (13,14), as well as enhanced immune status including improved cell-mediated immune response, serum thymulin activity, and IL-2 production, and increased response to skin-test antigens (12,15,42). Also, when cultures of white blood cells from elderly subjects were supplemented with 15 μM zinc (the physiologic concentration), they produced interferon-alpha in amounts comparable to those from the younger subjects (43). In a randomized, double-blind, placebo-controlled clinical trial (N=81), institutionalized elderly had a significant decrease in the mean number of respiratory infections 2 years following supplementation with micronutrients containing zinc and selenium, but not vitamins (44). In another, larger (N=725), randomized, double-blind, placebo-controlled intervention study, low-dose supplementation of zinc and selenium significantly increased the humoral response in institutionalized elderly after vaccination (45). The number without respiratory tract infections during the study was also found to be higher in elderly that received trace elements over a 2-year period (45). While these studies suggest a protective effect of zinc against respiratory infections, contribution from other nutrients present in the mixture cannot be ruled out.

The results from our current study, in addition to these earlier findings, suggest that elderly with low serum zinc concentrations might benefit from zinc supplementation. Such a measure has the potential to reduce not only the number of episodes and duration of pneumonia and the total and duration of antibiotic use due to pneumonia, but also overall deaths in the elderly. Randomized, double blind, controlled studies are needed to determine the efficacy of zinc supplementation as a potential low cost intervention to reduce morbidity and mortality due to pneumonia in this vulnerable population.

Acknowledgements

This work was supported by NIA, National Institutes of Health Grant 1R01-AG13975, and United States Department of Agriculture agreement 58−1950−9−001, and a grant for preparation of study capsules from Hoffmann-LaRoche Vitamins and Fine Chemicals Division (currently DSM) Inc. The funding organizations did not have input into the design or conduct of the study, into the collection, analysis, or interpretation of the data, or in the preparation, review, or approval of the manuscript. Dr. Meydani had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs. Meydani, Fine, Hamer and Ms. Leka were responsible for data acquisition. Drs. Meydani, Barnett, Dallal, Fine, Jacques and Hamer were responsible for the study concept and design, interpretation of study findings, and drafting of the manuscript. Dr. Dallal was responsible for statistical analyses. Drs Meydani, Barnett, Fine, Jacques and Hamer helped to direct these analyses. All authors were responsible for critical review and revision of the manuscript for important intellectual content, as well as final approval of the version to be published. All authors declare that we have no conflict of interest.

Sources of support: This work was supported by NIA, National Institutes of Health Grant 1R01- AG13975, United States Department of Agriculture agreement 58−1950−9−001, and a grant for preparation of study capsules from Hoffmann-LaRoche Vitamins and Fine Chemicals Division (currently DSM) Inc.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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Table 1

Characteristics of study subjects

Baseline characteristics	Participants with baseline serum zinc concentration (N=578)[χ]	Participants with final serum zinc concentration (N=420)[δ]	Participants with normal final serum zinc concentration (≥70ug/dL) (N=310)[ϕ]	Participants with low final serum zinc concentration (<70ug/dL) (N=110)[γ]
Age (y), mean (SD)	84.6 (7.6)	84.6 (7.5)	84.1 (7.1)	86.1 (7.7)
Females, n (%)	419 (72)	314 (75)	227 (73)	87 (79)
Caucasians, n (%)	544 (94)	398 (95)	291 (94)	107 (97)
Body mass index (kg/m^2), mean (SD)	25.8 (5.2)	26.0 (5.2)	26.0 (4.9)	26.0 (5.9)
Current smoker, n (%)	43 (8)	30 (7)	24 (8)	6 (6)
Vitamin E supplementation, n (%)	290 (50)	217 (52)	141 (45)	62 (56)
Serum albumin (g/dL), mean (SD)	3.76 (0.34)	3.77 (0.34)	3.80 (0.33)	3.69 (0.33)
Total cholesterol (mg/dL), mean (SD)	197 (46)	197 (45)	197 (46)	199 (43)
White Blood Count (cells/μL), mean (SD)	7.10 (2.30)	6.96 (1.78)	7.01 (1.81)	6.82 (1.69)
Total lymphocyte count (cells/μL), mean (SD)	2.12 (1.41)	2.10 (0.75)	2.15 (0.78)	1.98 (0.66)
Lymphocyte (%), mean (SD)	30.1 (9.3)	30.7 (9.0)	31.1 (9.1)	29.7 (8.8)
Pneumococcal vaccination, n (%)	53 (9)	46 (11)	33 (11)	13 (12)
Non-steroidal anti-inflammatory drug (NSAID) use, n (%)	212 (37)	158 (38)	120 (39)	38 (35)
Baseline medical conditions				
OLD, n (%)[†]	146 (25)	98 (23)	71 (23)	27 (25)
Coronary artery disease, n (%)	194 (34)	131 (31)	88 (28)	43 (39)
Congestive heart failure, n (%)	120 (21)	79 (19)	52 (17)	27 (25)
Hypertension, n (%)	297 (51)	222 (53)	162 (52)	60 (55)
Diabetes mellitus, n (%)	117 (20)	87 (21)	68(22)	19 (17)
Malignancy, n (%)	53 (9)	44 (10)	34 (11)	10 (9)
Dementia, n (%)[‡]	286 (49)	207 (49)	149 (48)	58 (53)

Comparison between those with low versus normal final serum zinc concentrations were conducted using Student's t-test for independent samples (continuous measures) and Fisher's exact test (categorical measures); p=0.014, and 0.042 between these groups for age, and coronary artery disease, respectively; all other variables were not found to be significant.

[†] obstructive lung disease (includes asthma, chronic obstructive pulmonary disease, and chronic bronchitis)

[‡] includes Alzheimer's disease

[χ]: N=563−578;

[δ]: N=412−420;

[ϕ]: N=305−310;

[γ]: N=105−110

FOI 26-3132 - Document 5

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Table 2

Pneumonia, antibiotic use and death by baseline serum zinc concentration

	Baseline Serum Zinc Groups *		Rate Ratio or Mean Difference (95% CI)†	*p*
	≥70ug/dl (N=379)	<70ug/dl (N=174)		
Incidence of pneumonia (no. per person-yr)	0.34	0.37	0.87 (0.63, 1.21)	0.414
Duration of pneumonia (days per person-yr)	4.32	5.65	−1.5 (−3.4, 0.4)	0.126
Antibiotic prescriptions for pneumonia (no. per person-yr)	0.34	0.36	0.90 (0.65, 1.25)	0.539
Duration of antibiotic use for pneumonia (days per person-yr)	3.07	3.58	−0.7 (−2.0, 0.7)	0.330
Deaths due to pneumonia (no. per person-yr)	0.02	0.04	0.47 (0.15, 1.49)	0.198
Overall deaths (no. per person-yr)	0.12	0.19	0.61 (0.37, 1.00)	0.049

*: Crude values

†: Poisson regression analyses were used for incidence of pneumonia and number of antibiotic prescriptions, least squares regression analyses for duration of pneumonia and of antibiotic use, and Cox proportional hazard regression for deaths. All analyses controlled for treatment (supplemented with vitamin E or not), age, sex, chronic obstructive lung disease, current smoking, diabetes mellitus, year of enrollment (1998−2000) and baseline BMI; additionally controlling for coronary artery disease, and, in separate models, baseline serum albumin concentrations, did not affect the observed associations. *p* values derived from Poisson, least squares and Cox proportional hazard regression analyses.

FOI 26-3132 - Document 5

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Table 3

Pneumonia and antibiotic use by final serum zinc concentration

	Final Serum Zinc Groups*		Rate Ratio or Mean Difference (95% CI) †	
	≥70ug/dl (N=310)	<70ug/dl (N=110)		*p*
Incidence of pneumonia (no. per person-yr)	0.25	0.46	0.52 (0.36, 0.76)	<0.001
Duration of pneumonia (days per person-yr)	3.19	6.82	−3.9 (−6.2, −1.6)	<0.001
Antibiotic prescriptions for pneumonia (no. per person-yr)	0.26	0.48	0.52 (0.36, 0.75)	<0.001
Duration of antibiotic use for pneumonia (days per person-yr)	2.50	4.85	−2.6 (−4.4, −0.9)	0.004

*: Crude values

†: Poisson regression analyses were used for incidence of pneumonia and number of antibiotic prescriptions and least squares regression analyses for duration of pneumonia and of antibiotic use. All analyses controlled for treatment (supplemented with vitamin E or not), age, sex, chronic obstructive lung disease, current smoking, diabetes mellitus, year of enrollment (1998−2000) and baseline and change in BMI between baseline and follow-up; additionally controlling for coronary artery disease, and, in separate models, baseline serum albumin and change in serum albumin concentrations between baseline and follow-up, did not affect the observed associations. *p* values derived from Poisson and least squares regression analyses.

FOI 26-3132 - Document 5

COMMUNICATIONS BIOLOGY

ARTICLE

https://doi.org/10.1038/s42003-020-01577-x OPEN

Identification of 3-chymotrypsin like protease (3CLPro) inhibitors as potential anti-SARS-CoV-2 agents

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Emerging outbreak of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection is a major threat to public health. The morbidity is increasing due to lack of SARS-CoV-2 specific drugs. Herein, we have identified potential drugs that target the 3-chymotrypsin like protease (3CLpro), the main protease that is pivotal for the replication of SARS-CoV-2. Computational molecular modeling was used to screen 3987 FDA approved drugs, and 47 drugs were selected to study their inhibitory effects on SARS-CoV-2 specific 3CLpro enzyme in vitro. Our results indicate that boceprevir, ombitasvir, paritaprevir, tipranavir, ivermectin, and micafungin exhibited inhibitory effect towards 3CLpro enzymatic activity. The 100 ns molecular dynamics simulation studies showed that ivermectin may require homodimeric form of 3CLpro enzyme for its inhibitory activity. In summary, these molecules could be useful to develop highly specific therapeutically viable drugs to inhibit the SARS-CoV-2 replication either alone or in combination with drugs specific for other SARS-CoV-2 viral targets.

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FOI 26-3132 - Document 5

The major pandemic outbreak of the 21st century due to severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has become a global threat to public health because of its high rate of infection leading to mortality. As of 24 December 2020, there are a total of 13,881,620 COVID-19 positive cases and 272,820 deaths in the United States alone and 64,326,880 confirmed cases and 1,488,992 deaths globally (https://coronavirus.jhu.edu/). The death toll is increasing at an alarming rate because of the lack of COVID-19 specific drugs or vaccines. Development, validation, and approval of COVID-19 specific drugs takes years[1]. Therefore, the idea of drug repositioning, also known as repurposing, is an important strategy to control the sudden outbreak of life-threatening infectious agents that spread rapidly. FDA approved anti-viral drugs are known to be safe for use in humans[2], but their effectiveness against SARS-CoV-2 needs to be experimentally validated. Several FDA approved anti-viral drugs such as favipiravir, danoprevir, darunavir, lopinavir, oseltamivir, ritonavir, remdesivir, and umifenovir are in clinical trials to study anti-COVID-19 activity[3]. However, the effectiveness of these drugs for preventing or reducing the severity of symptoms of COVID-19 has not yet been completely established. Therefore, there is an urgent need to identify additional drug candidates to target different SARS-CoV-2 proteins for enhanced efficacy in the treatment of COVID-19.

Recently, Wu et al.[4] sequenced and compared SARS-CoV-2 genome with other coronaviruses (CoVs) and confirmed that novel SARS-CoV-2 belongs to β-CoVs, which were originally found in bats and have now adapted to infect humans. CoVs are RNA viruses with positive-sense single stranded RNA (+ssRNA) as their genetic material[5] and recent studies have shown that SARS-CoV-2 shares ~89% sequence similarity with other SARS-CoVs[4]. Additionally, SARS-CoV-2 has similar genetic organization as other SARS-CoVs with a 5′-untranslated region followed by 16 non-structural proteins (open reading frame; ORF1a and ORF1b complex) also called as replicase complex, and the structural proteins such as spike (S), envelop (E), membrane (M), and nucleocaspid (N) protein along with other accessory proteins present towards the 3′ end[6]. The life cycle of the virus begins with the binding of the S protein of the virus to its receptor on the host cells, the ACE2. The binding is then followed by the fusion of the viral envelop with host cell membrane and the release of the viral genome into the cytoplasm[7]. The viral genome (+ssRNA) hijacks the host ribosomes and gets translated into ~ 800KDa large polypeptide (PP) chain. The newly generated PP chain is auto-proteolytically cleaved by two proteases such as papain like proteases (PLpro) and 3-chyomotrypsin like protease (3CLpro), encoded by the viral genome, to generate several non-structural proteins (NSPs) required for the viral replication. 3CLpro is also called the main protease (Mpro) and plays a major role in the viral replication. PLpro and 3CLpro cleaves the PP chain into 16 NSPs and out of the 16 NSPs generated, 11 NSPs are generated by the 3CLpro, making this protease one of the major targets for developing anti-SARS-CoV drugs[8,9]. On the other hand, structural and other accessary proteins are generated through a unique mechanism called sub-genome (Sg) translation. Sg's are produced through discontinuous transcription from 5′ end of the anti-sense viral RNA[10–13]. After successful genome replication and translation, NSPs, structural proteins and accessory proteins assemble along with positive-sense viral RNA genome to form a new virion. The CoVs genome and proteolytic cleavages by PLpro and 3CLpro is illustrated in Fig. 1.

The genomic and protein sequences for SARS-CoV-2 are publicly available from the NIH gene data bank[4,6,14]. Herein, we have selected 3CLpro of SARS-CoV-2 as a target to identify potential inhibitors since this protease is indispensable for viral replication and hence an excellent drug target[9]. The structure of 3CLpro protein of SARS-CoV-2 in complex with an inhibitor N3 is available in the PDB database (ID: 6LU7). To identify the FDA approved drugs as inhibitors for 3CLpro, in silico drug screening studies were carried out. In all, 3987 FDA approved drugs (SuperDrugs2 database) were sorted as viral protease inhibitors (PIs), viral non-protease inhibitors (VNIs) and off-target drugs (OTDs), and screened for the anti-3CLpro activity using the Molecular Operating Environment (MOE) software. The protein structure-based drug design using computational methods is an alternative for screening of currently approved drugs to rapidly identify potential drug candidates for the treatment of emerging infectious diseases such as COVID-19[15–17]. However, the potential for false positives with computational modeling is one of the most common limitation of docking studies[18]. Therefore, we have established SARS-CoV-2 3CLpro enzymatic assays for selected drugs using commercially available 3CLpro protease inhibitor screening assay kits to evaluate the in vitro inhibitory activity of the drugs and investigated whether any correlation exist between the computational binding score and the in vitro inhibitory activity. In this report, we have selected 47 from the list of 3987 FDA approved drugs based on binding score, side effects, half-life, active form, immunosuppressant properties, autofluorescence, and availability for an in vitro 3CLpro enzymatic inhibitor-screening assay. We observed that, boceprevir, ombitasvir, paritaprevir, tipranavir, and micafungin exhibited partial inhibitory effect whereas, ivermectin blocked more than 85% of 3CLpro activity of SARS-CoV-2. Although the anti-viral activity of ivermectin mediated through the blocking of α/β1 importin[19–23] is established, herein we report the inhibitory effects of ivermectin on 3CLpro enzyme of SARS-CoV-2, suggesting additional anti-viral mechanism of ivermectin towards SARS-CoV-2.

Results

In silico screening of FDA approved drugs for potential binding to SARS-CoV-2 3CLpro enzyme. All the 3987 FDA approved drugs (downloaded from SuperDrugs2) were sorted as PIs, VNIs, and OTDs and docked with monomeric form of 3CLpro protein using the Molecular Operating Environment (MOE) software. Drugs were ranked according to the highest binding energy (S score). S score was calculated using the London dG score for placement and GBVI/WSA dG score for refinement of poses. A binding energy with ≤−6.5 kcal/mol (S score) was selected as a cutoff to rank the drugs with highest binding affinity[16]. The list was further narrowed down based on the criteria listed in the Methods section. We found that 56 drugs have a binding affinity of <−6.50 kcal/mol for the 3CLpro enzyme (Table 1). The computational study suggested that the list of drugs identified might inhibit the SARS-CoV-2 viral replication by targeting the viral 3CL protease. However, the potential for false positives with the predicted binding score is one of the most common limitation of docking studies[24,25]. Hence, to rule out any ambiguity in false prediction, we carried out the in vitro enzymatic assay to identify potential therapeutics and investigate correlation between the binding score and the in vitro activity. In the current study, we did not find any correlation between the in vitro results of selected drugs and their computational inhibition constants. Even though, computational studies are being widely used to predict the initial protein-drug interactions, in vitro screening of the drugs is necessary to confirm the inhibitory activities of the drugs.

Inhibition of SARS-CoV-2 3CLpro enzymatic activity by selected drug candidates. To further validate the SARS-CoV-2 3CLpro inhibitory activity of the selected drugs from computational studies, we performed an in vitro enzymatic inhibitory

FOI 26-3132 - Document 5

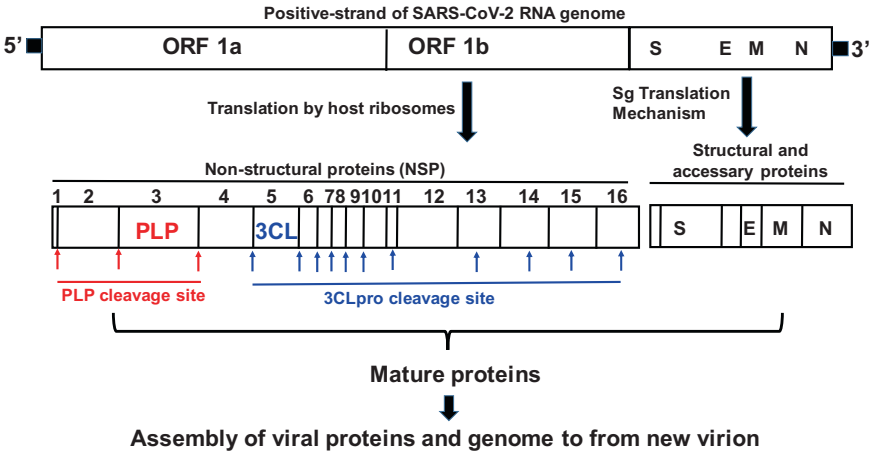


Fig. 1 Representation of SARS-CoV-2 genome arrangement and protease cleavage sites. Genetic material (RNA) of SARS-CoV-2 enters the host cell and borrows ribosome to translate into 16 nonstructural proteins upon auto-proteolytic cleavage by PLpro and 3CLpro enzymes. Structural proteins (S, E, M, and N) are translated by sg translation mechanism. All mature proteins assemble along with positive-sense single stranded RNA genome to form new virion. Arrows indicates the protease cleavage sites. ORF open reading frame, PLP papain like protease, 3CLP 3-chymotrypsin like protease, S spike protein, E envelop protein, M membrane protein, N nucleocaspid protein.

assay using commercially available assay kits. The background autofluorescence of the selected 56 compounds was measured by excitation/emission wavelength of 360/460 nm. Nine out of 56 drugs exhibited extremely high autofluorescence (Supplementary Fig. 2) and were therefore eliminated from the in vitro protease inhibitor enzymatic assay. The remaining 47 drugs were sorted as PI, VNIs, and OTDs. The specific mechanism of action and their clinical uses are listed in Supplementary Table 1. The 3CLpro inhibitory activity was screened at 50 μM concentration of the drugs. Among the 17 PIs screened, boceprevir, paritaprevir and tipranavir significantly inhibited the 3CLpro enzymatic activity by 45, 70, and 64%, respectively (Fig. 2). Out of the 17 VNIs, only ombitasvir was able to partially block (65%) the 3CLpro enzymatic activity (Fig. 3). Interestingly, as shown in Fig. 4, out of 13 OTDs only ivermectin completely blocked (>80%) the 3CLpro activity at 50 μM concentration. Additionally, micafungin exhibited partial inhibitory activity (57%) against 3CLpro of SARS-CoV-2.

The compounds that exhibited more than 50% inhibitory activity were subjected to subsequent dose-dependent studies to calculate the concentration required to inhibit 50% of the 3CLpro enzymatic activity (IC_{50}). Boceprevir, ivermectin, micafungin, ombitasvir, paritaprevir, and tipranavir were subjected to dose-dependent inhibitory activity studies. As shown in the Fig. 5, ivermectin inhibited more than 85% of the enzymatic activity at 50 μM concentration, whereas micafungin and paritaprevir inhibited around 80% of the enzymatic activity at 100 μM concentration. Both tipranavir and ombitasvir were able to inhibit only 50% of the enzymatic activity even at 100 μM concentration (Fig. 5). The percent enzymatic activity versus the log concentration of the inhibitors was used to calculate the IC_{50} values using non-linear curve fit model as described under Methods section. The calculated IC_{50} values for ivermectin, tipranavir, boceprevir, micafungin, paritaprevir, and ombitasvir were found to be 21.5, 27.7, 31.4, 47.6, 73.4, and 75.5 μM, respectively (Table 2). Taken together, these studies suggest that the molecules listed above exhibited inhibitory activity against 3CLpro enzyme of SARS-CoV-2.

Structural interaction of boceprevir, paritaprevir ombitasvir, tipranavir, ivermectin, and micafungin with the enzymatic active site of 3CLpro. The presence of an unconventional catalytic cysteine residue in 3CLpro makes it unique as compared to other chymotrypsine like enzymes and other Ser (or Cys) hydrolases[26]. In addition, the 3CLpro consist of a catalytic Cys^{145}-His^{41} dyad instead of a canonical Ser(Cys)-His-Asp(Glu) triad[27]. This catalytic dyad is activated in the presence of the substrate containing Leu-Gln↓Ser-Ala-Gly (↓ marks the cleavage site)[26]. Structurally the catalytic residues Cys^{145} and His^{41} in 3CLpro are buried in an active site cavity located on the surface of the protein (Supplementary Fig. 1) and both residues are at a distance of 3.8 Å[26,28]. This distance might be long enough to prevent formation of any intermolecular hydrogen bond between Cys^{145} and His^{41} at physiological pH. Additionally, the Cys^{145} is protonated at physiological pH and His^{41} is present in the neutral state. The substrate binding triggers the intramolecular proton transfer from Cys^{145} to His^{41} triggered via the attack of Cys^{145}-Sulfur onto the (C=O) of the substrate peptide bond[27]. During catalytic activity, the protonated His^{41} is stabilized by the presence of H_2O molecule close to His^{41}. Thus, the ability to form a hydrogen bond with both Cys^{145} and His^{41} residues or either with one of these residues might be a characteristic of a good inhibitor.

Using computational modeling, the structural interaction of boceprevir, paritaprevir ombitasvir, tipranavir, ivermectin, and micafungin with 3CLpro SARS-CoV-2 enzyme was studied. We found that carbonyl (C=O) group in ivermectin and boceprevir forms a hydrogen bond with Cys^{145} which might explain their inhibitory activity (Fig. 6a, e; panel I and II). Similar type of hydrogen bonding was also observed between carbonyl (C=O) group in paritaprevir next to the sulfonamide and Cys^{145} and also between sulfonamide group in tipranavir and Cys^{145} (Fig. 6b, c: panel I and II). In addition, same carbonyl (C=O) group in paritapavir also shows hydrogen bonding interaction with His^{41} (Fig. 6b; panel I and II). However, ombitasvir and micafungin show a hydrogen bonding interaction with Glu^{166} (Fig. 6d, f; panel I and II). The hydrogen bonding interaction of Glu^{166} with ombitsavir and micafungin is very important, as the Glu^{166} is responsible for the formation of the homodimer of 3CLpro in SARS-CoV-2[28]. This dimer form is important for its enzymatic activity and any interaction with Glu^{166} can result in the formation of an inactive monomer which interferes with the enzyme activity of 3CLpro[28]. Computational modeling illustrations are presented in Fig. 6a–f; panel-III shows the interaction of the drugs with the lipophilic pocket of the 3CLpro enzymatic site.

FOI 26-3132 - Document 5

Table 1 List of all the drugs with highest S score for 3CL protease calculated by computational studies (S score).

SL. No.	Drug	Binding affinity (Kcal/mol) to 3CLpro
1	Abacavir	−6.6178
2	Amprenavir	−8.29197
3	Asunaprevir	−9.64249
4	Atazanavir	−8.51517
5	Atrovastatin	−7.98771
6	Beclabuvir	−8.45563
7	Boceprevir	−8.4209
8	Camostat	−7.66508
9	Candicidin	−9.05496
10	Chloroquine	−6.7514
11	Daclatasvir	−8.47852
12	Danoprevir	−9.26908
13	Darunavir	−8.59002
14	Delavirdine (mesylate)	−7.3092
15	Elbasvir	−8.77218
16	Elvitegravir	−7.48581
17	Etravirine	−6.91494
18	Favipiravir	−4.23592
19	Fumagillin	−7.25118
20	Gabexate	−7.24702
21	Glecaprevir	−8.70897
22	Grazoprevir	−8.85946
23	Hydroxychloroquine	−7.03572
24	Indinavir	−8.39968
25	Itraconazole	−7.99098
26	Ivermectin	−7.74053
27	Ledipasvir	−9.52825
28	Lopinavir	−9.10074
29	Maraviroc	−8.19033
30	Methylprednisolone	−6.42667
31	Micafungin	−9.60339
32	Nelfinavir	−8.55575
33	Ombitasvir	−8.96571
34	Oseltamvir Phosphate	−7.04549
35	Paritaprevir	−7.43470
36	Peramivir	−6.7811
37	Pibrentasvir	−9.87502
38	Pimodivir	−6.79098
39	Pleconaril	−7.16664
40	Posaconazole	−7.95234
41	Quinine	−6.67637
42	Raltegravir	−7.76854
43	Remdesivir	−5.9488
44	Ribavirin	−6.3251
45	Ribostamycin	−7.28802
46	Rilpivirine	−7.09415
47	Ritonavir	−9.94965
48	Saquinavir	−9.25575
49	Simeprevir	−9.50916
50	Sofosbuvir	−8.4513
51	Telaprevir	−8.96481
52	Temsavir	−8.33862
53	Tenofovir Diphosphate	−6.557
54	Tipranavir	−7.47819
55	Umifenovir	−7.23012
56	Velpatasvir	−9.33025

100 nanosecond (ns) Molecular Dynamics simulations for micafungin and ivermectin. To investigate the stability of these docking poses, 100 ns molecular dynamics (MD) simulation studies were performed for two compounds, ivermectin and micafungin. Figure 7a panel I–III shows the MD simulations of micafungin with the monomer form of 3CLpro. The MD simulation data suggests that micafungin was stable and remained bound in the active site pocket throughout the 100ns simulated time (compare Fig. 7a I–III). The analysis of protein-ligand interaction fingerprints between the monomeric 3CLpro enzyme and micafungin (Fig. 7a, panel-IV) shows that micafungin has a predominant interaction with Glu^{166}. It is possible that the micafungin remained bound in the pocket of the monomeric form of 3CLpro for the entire length of the 100-ns trajectory via a hydrogen bonding with Glu^{166}. As noted earlier, interaction with Glu^{166} suggests interference with the dimerization of the 3CLpro in SARS-CoV-2, which is required for its activity[28] thus explaining the inhibitory activity of micafungin against 3CLpro enzyme. Supplementary Movie 1 presents the real-time interaction of micafungin with the active site of monomeric 3CLpro indicating the stability of micafungin in the catalytic pocket of monomer.

The MD simulations for ivermectin are shown in Fig. 7b (panel II–III), where ivermectin diffuses out of the catalytic pocket of 3CLpro monomer after 85 ns. It is evident from the protein-ligand fingerprint map that ivermectin interacts with both Cys^{145} and His^{41} of the 3CLpro monomer for about 14 ns (Fig. 7b, panel-IV). Later, ivermectin loses its interaction with His^{41} and does not show interaction with any amino acids of interest (Cys^{145}, His^{41}, and Glu^{166}) and eventually diffuses out of the pocket at 85 ns (Fig. 7b, panel-II). Supplementary Movie 2 shows the instability of the ivermectin in the catalytic pocket of the monomeric form of 3CLpro. Since the homodimer is the active form of 3CLpro enzyme[28], we hypothesized that the homodimeric form of 3CLpro is required to stabilize ivermectin in the catalytic pocket and hence is responsible for the inhibitory activity of ivermectin. To test our hypothesis, MD simulations of ivermectin with the homodimer form of 3CLpro was performed. Interestingly, we observed that ivermectin remained bound in the catalytic pocket of the homodimer (compare Fig. 7c, panel I–III) throughout the period of the simulation. The detailed analysis of the homodimer 3CLpro-ivermectin fingerprint region (Fig. 7c, panel-IV) shows that ivermectin interacts with both Cys^{145} and His^{41} for 2 ns, then with His^{41}. After 85 ns, ivermectin contacts with Ser^{1} of the neighboring monomer, suggesting that this amino acid residue assists in the stabilization of ivermectin in the catalytic binding pocket (Fig. 7c, panel IV). Supplementary Movie 3 exhibits the real-time MD simulation interaction and the stability of ivermectin in the catalytic pocket with homodimer of 3CLpro from 0–100ns.

Further, Supplementary Fig. 3 shows the binding affinity (S-score) over the course of the MD simulation for ivermectin with the monomer and homodimer form of 3CLpro, and micafungin with monomer form of 3CLpro. We observed that the S-score for micafungin was stable over the period of computation whereas, ivermectin with monomer form of 3CLpro fluctuated from −9.64 to −2.2 kcal/mol. As shown in the Supplementary Fig. 3, upon leaving the active site ~85 ns ivermectin exhibited an increase in S-score after (~−2.0 kcal/mol). This is in stark contrast to the S-score of ivermectin in complex with the homodimer form of 3CLpro, which remained stable throughout the simulation with an average of −5.64 kcal/mol. Taken together, this computational model provides a framework for the possible interaction between these inhibitors and 3CLpro. However, the structural interaction of these drugs with SARS-CoV-2 3CLpro needs to be validated by X-ray crystallographic studies.

Discussion

COVID-19 is a disease caused by the SARS-CoV-2 and is a major threat to public health globally because of the high rates of infection and mortality. Morbidity and mortality continue to rise

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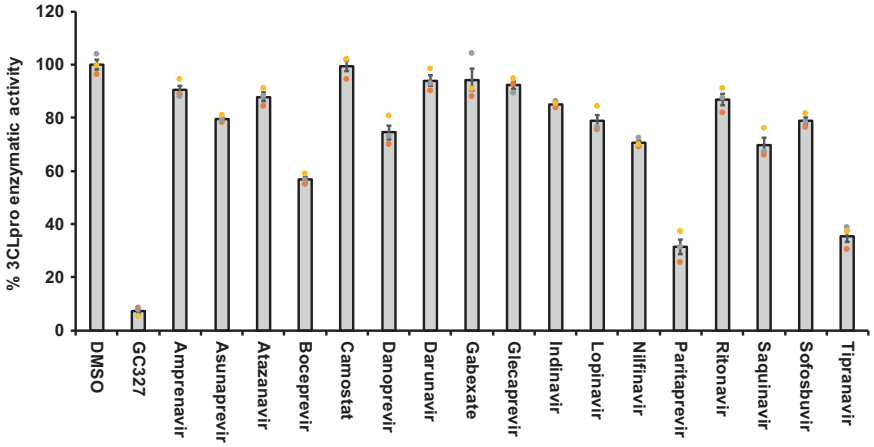


Fig. 2 Protease inhibitors boceprevir, paritapravir, and tipranavir exhibited partial inhibitory activity against SARS-CoV-2 3CLpro enzyme. The selected FDA approved viral-protease inhibitors were screened for their inhibitory activity against SARS-CoV-2 3CLpro enzyme as described under Methods section. The fluorescence units were converted to percent enzymatic activity considering DMSO treated control as 100% activity. Blank values were subtracted from all the readings before calculating the percent activity. Representative of three individual experiments with triplicate values were presented graphically ($n = 3$). P value < 0.001 considered as statistically significant. One-way ANOVA with Dunnett's Multiple Comparison post-hoc test was used to calculate the statistical significance.

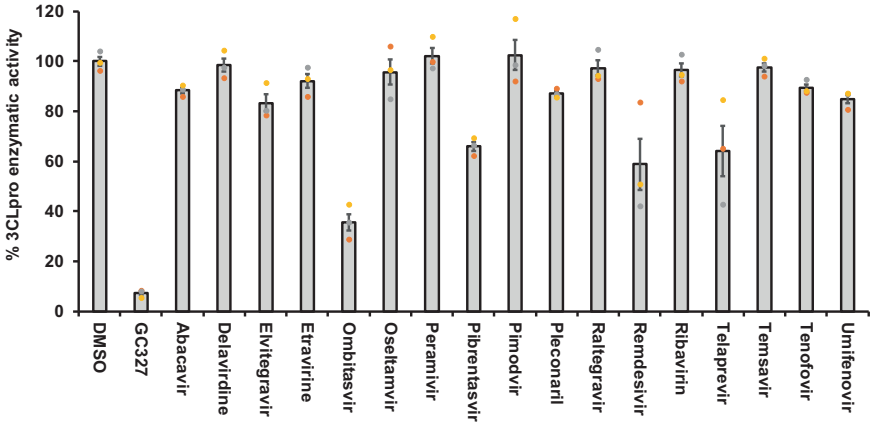


Fig. 3 Non protease inhibitor ombitasvir inhibited SARS-CoV-2 3CLpro activity partially. The non-protease anti-viral drugs selected by computational studies were screened for their inhibitory activity against SARS-CoV-2 3CLpro enzyme as described under Methods section. The percent enzymatic activity was calculated as described in Fig. 1 legend. Blank values were subtracted from all the readings before calculating the percent activity. Representative of three individual experiments with triplicate values were presented graphically ($n = 3$). P value < 0.001 considered as statistically significant. One-way ANOVA with Dunnett's Multiple Comparison post-hoc test used to calculate the statistical significance.

due to the lack of a specific vaccine and drugs that prevent COVID-19 disease progression. There is an urgent need to identify and test potential therapeutics for this disease. One approach that may lead to a more rapid increase in treatment options is to repurpose currently approved FDA drugs for their ability to prevent or reduce the spread of virus and severity of COVID-19 pathogenesis. Many of the FDA approved drugs are being repurposed in clinics to treat COVID-19. However, the effectiveness of these drugs and their specific targets for preventing or reducing the severity of symptoms of COVID-19 has not yet been completely established[2,29–31]. Therefore, several laboratories are identifying specific drugs for many targets of SARS-CoV-2. Herein, we have investigated 47 FDA approved drugs that inhibit the SARS-COV-2 3CLpro enzymatic activity, the main enzyme for viral replication and the preferred drug target for COVID- 19[32]. We used MOE computational studies for the initial screening to select the drugs that have high affinity for 3CLpro and further functional inhibitory activity of the 47 selected drugs was confirmed using in vitro enzymatic assay. As noted in the previous studies[24,25], our data suggests that inhibitory effects of drugs predicted from the computational screening as defined by the S-score do not agree with our experimental in vitro studies. Thus, additional in vitro screening for all the drugs is warranted.

Among 17 PIs tested here, boceprevir, paritaprevir, and tipranavir were able to partially inhibit the 3CLpro enzymatic activity at 50 µM drug concentration (Fig. 2). It has been shown that boceprevir and paritaprevir inhibit the hepatitis-C virus by inhibiting protease activity of nonstructural protein 3 and 4A (NS3/4A)[33,34]. Tipranavir is a retroviral protease inhibitor that binds to active site of HIV protease and prevents proteolytic cleaving of precursor polyproteins into mature functional proteins thereby inhibiting the viral replication[35]. Apart from these drugs, known PIs lopinavir and ritonavir did not exhibit any 3CLpro inhibitory activity (Fig. 2); thus explaining the ineffectiveness of these drugs in clinical trials of COVID-19 treatment[31]. Our observation with lopinavir and ritonavir are in agreement with Ma et al.[34]. Further, the VNI agent such as ombitasvir which targets the nonstructural proteins (NS5A) of hepatitis-C virus to inhibit the viral replication and assembly[36] was also able to

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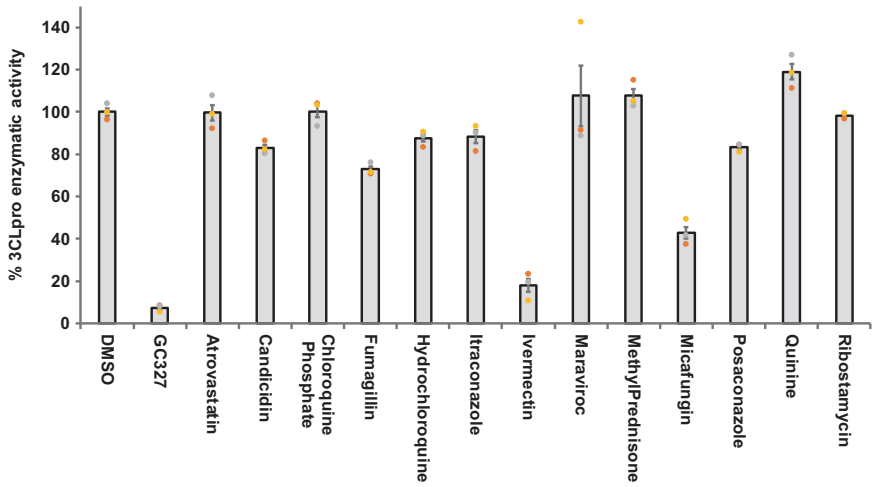


Fig. 4 Ivermectin exhibited complete inhibition of SARS-CoV-2 3CLpro enzymatic activity whereas micafungin partially inhibited the enzyme. The off-target drugs that are being used to treat non-viral ailments selected by in silico studies were screened for their inhibitory activity against SARS-CoV-2 3CLpro enzyme as described under Methods section. The percent enzymatic activity was calculated as described in Fig. 1 legend. Blank values were subtracted from all the readings before calculating the percent activity. Representative of three individual experiments with triplicate values were presented graphically ($n = 3$). P value < 0.001 considered as statistically significant. One-way ANOVA with Dunnett's Multiple Comparison post-hoc test used to calculate the statistical significance.

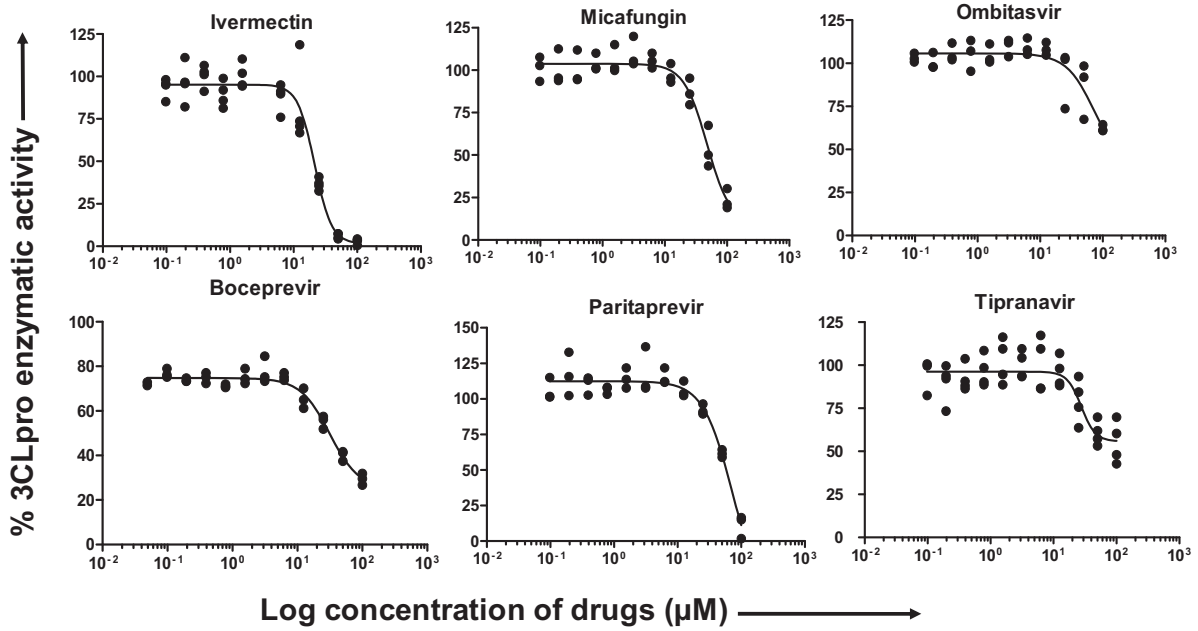


Fig. 5 Dose-dependent inhibition of SARS-CoV-2 3CLpro activity by selected PIs, VNIs, and OTDs. The drug candidates that exhibited more than 50% of inhibitory activity at 50 μM concentration were selected for dose-dependent and IC_{50} calculation studies. A serial dilution of drugs ranging from 0 to 100 μM in assay buffer was used. The percent activity was calculated as described in Fig. 1 legend. Representative of three individual experiments with triplicate values were presented graphically ($n = 3$). Non-linear regression (curve fit) with four variable dose vs inhibition was used to calculate the IC_{50} values using GraphPad Prism.

Table 2 IC_{50} values of non-viral protease inhibitor drugs for SARS-CoV-2 3CL protease.

SL. No.	Drug name	IC_{50} values
1	Ivermectin	21.53
2	Tipranavir	27.66
3	Boceprevir	31.36
4	Micafungin	47.63
5	Paritaprevir	73.38
6	Ombitasvir	75.49

partially inhibit the 3CLpro enzymatic activity in vitro. In addition to the anti-viral drugs, micafungin, one of the OTDs tested, also inhibited 66% of 3CLpro activity. Micafungin is a broad spectrum anti-fungal drug which belongs to the class of echinocandin and acts by targeting fungal β-1–3 glucan synthase[37].

While the reason for the partial inhibitory effect of the agents boceprevir, ombitasvir, paritaprevir, tipranavir, and micafungin towards 3CLpro is not clearly understood, it is possible that the strength of the hydrogen bonding interactions between these agents and Cys^{145}/His^{41}/Glu^{166} of 3CLpro may explain differential inhibitory effect. Nonetheless, our studies provide avenues

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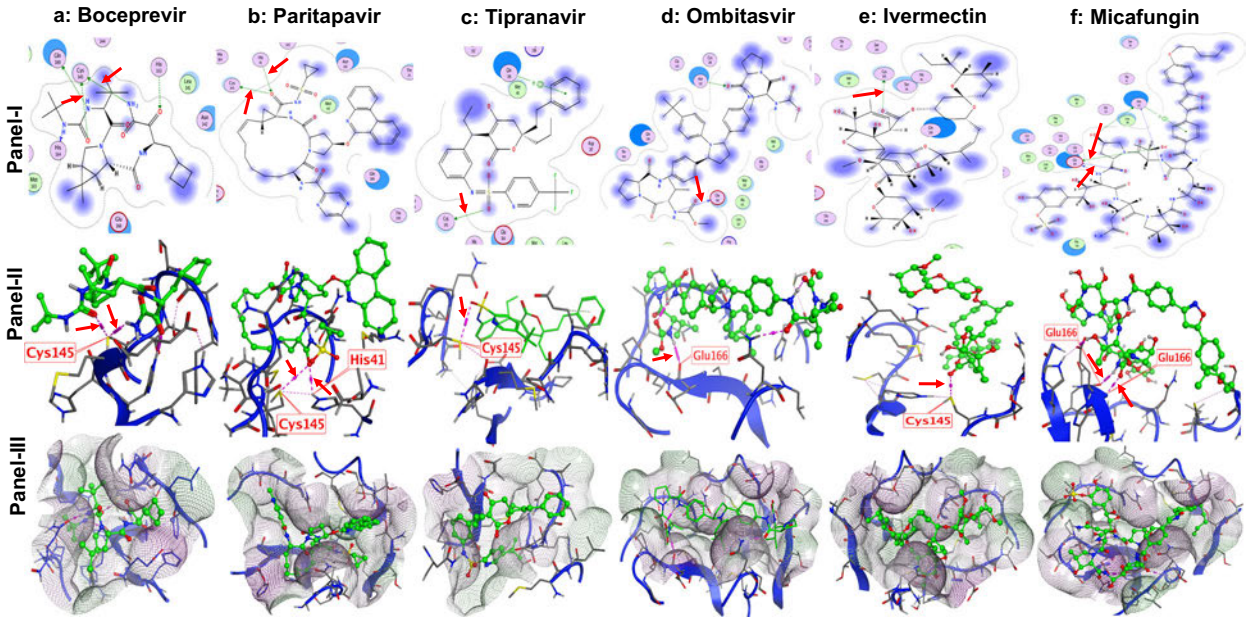


Fig. 6 Structural analysis and interaction of the potential inhibitors with SARS-CoV-2 3CLpro enzyme. Inhibitors were docked with active site of 3CLpro protein. Panel-I: ligand interaction map. Panel-II: interaction of inhibitors specific amino acids of 3CLpro at active site. Panel-III: lipophilic cavity of active site with drugs interacting with specific amino acids. Boceprevir (**a**), partapravir (**b**), tipranavir (**c**), ombitasvir (**d**), ivermectin (**e**), and micafungin (**f**) are arranged in columns for comparison. Drugs are represented in green color with ball and stick model. Arrows indicate the C–H, N–H, and C–O bonds between drugs and with Cys^{145}, His^{41}, and Glu^{166} residues since they are essential for the enzymatic activity of 3CLpro enzyme. However, we also observed the drugs interacting with neighboring amino acid residues.

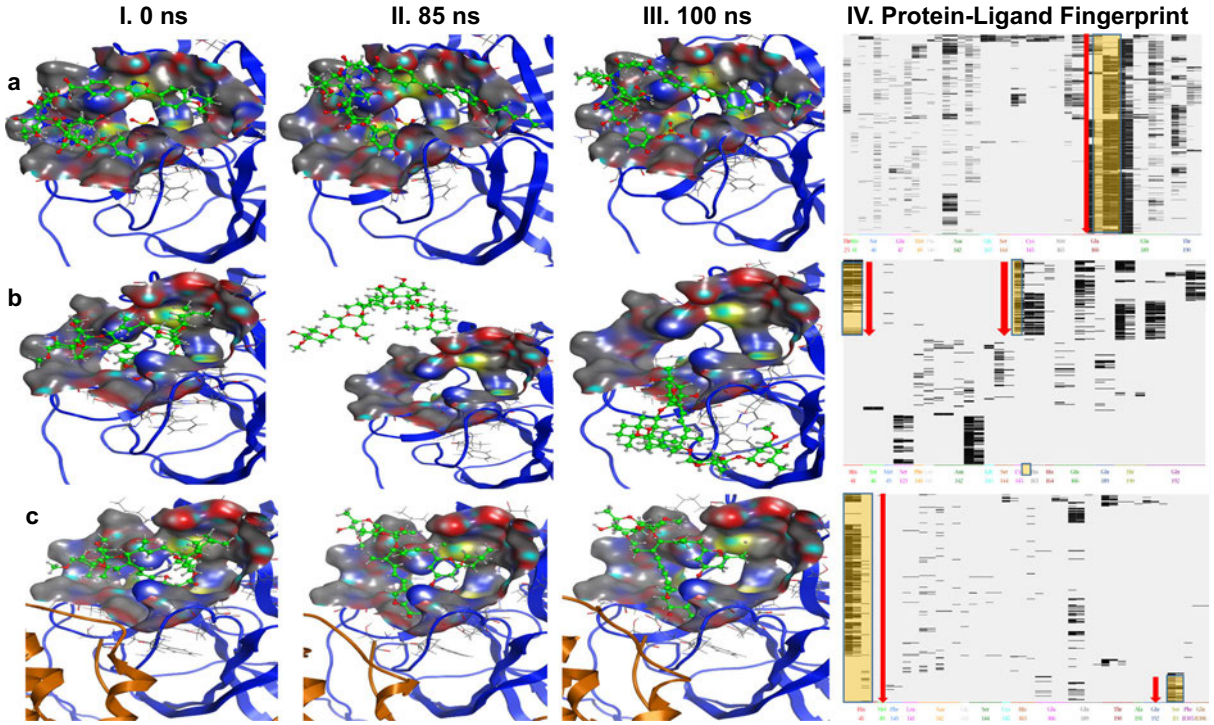


Fig. 7 MD 100 ns simulation studies for ivermectin and micafungin. MD simulation studies were carried as described under Methods section. Interaction of micafungin (**a**), and ivermectin (**b**) with monomeric form of 3 CLpro enzyme. **c** Interaction of ivermectin with active site of 3CLpro homodimer. Panel I–III represents the interaction of ligand at different time points in nano second (ns). Panel-IV represents the ligand-binding fingerprint of micafungin and ivermectin with specific amino acids of 3CLpro enzymes.

to use the scaffold of these molecules to design highly potent and specific inhibitors for SARS-CoV-2 3CLpro enzyme.

Interestingly, one of the OTD, ivermectin was able to inhibit more than 85% (almost completely) of 3CLpro activity in our in vitro enzymatic assay with an IC_{50} value of 21 μM. These findings suggest the potential of ivermectin to inhibit the SARS-CoV-2 replication. In support of this, a recent finding suggested that ivermectin (5 μM) inhibited the replication of live

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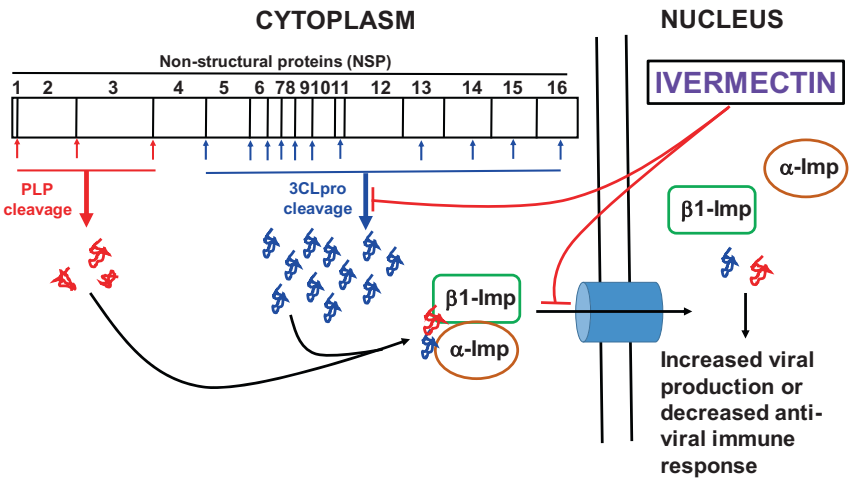


Fig. 8 Schematic representation of anti-viral mechanism of ivermectin. Hypothetical model illustrating the inhibition of SARS-CoV-2 replication by ivermectin mediated through the blocking of α/β1-importin (imp) as well as 3CLpro enzymatic activity.

SARS-CoV-2 isolated from Australia (VIo1/2020) in Vero/hSLAM cells[23]. They found that >5000-fold viral counts were reduced in 48 hr in both culture supernatant (release of new virion: 93%) as well as inside the cells (unreleased and unassembled virion: 99.8%) when compared to DMSO treated infected cells. Interestingly, this study reported the IC_{50} value of ivermectin as 2.5 μM[23], whereas, we observed an IC_{50} value of 21 μM (10-fold increase). The variability in the IC_{50} values reported could be attributed towards the differences in the assay conditions such as the use of live virus vs enzymatic assay with purified 3CLpro protein. Further, preclinical studies need to be established to validate the in vivo inhibitory activity and IC_{50} values of ivermectin.

Ivermectin is known to be effective against many positive-sense, single stranded RNA viruses such as Zika, Dengue, Yellow fever, West nile, Venezuelan equine encephalitis, Chikungunya, Semliki forest, Sindbis, Rorcine Reproductive and Respiratory Syndrome, and Human immunodeficiency-1 viruses[38]. The list of anti-viral effects of ivermectin against other RNA and DNA viruses were summarized in a recent review[38]. Earlier studies have demonstrated that the possible anti-viral mechanism of ivermectin was through the blockage of viral-protein transportation to the nucleus by inhibiting the interaction between viral protein and α/β1 importin heterodimer, a known transporter of viral proteins to the nucleus especially for RNA viruses[19–23]. However, in this study, we have reported that ivermectin inhibits the enzymatic activity of SARS-CoV-2 3CLpro and thus may potentially inhibit the replication of RNA viruses including SARS-CoV-2. These studies suggest that ivermectin could be a potential drug candidate to inhibit the SARS-CoV-2 replication and the proposed anti-viral mechanism of ivermectin presented in Fig. 8 and in vivo efficacy of ivermectin towards COVID-19 is currently been evaluated in clinical trials (ClinicalTrials.gov Identifier: NCT04438850).

In conclusion, the SARS-CoV-2 specific 3CLpro enzyme was used as a target to screen the potential drugs that have high binding affinity for 3CLpro since it plays a major role during viral replication. We have identified that boceprevir, micafungin, ombitasvir, paritaprevir, and tipranavir exhibited partial inhibitory effect, whereas ivermectin was able to completely inhibit the SARS-COV-2 3CLpro enzymatic activity in vitro at the tested doses. The 100 ns MD simulation studies suggest that the ivermectin may require homodimeric form of 3CLpro enzyme for its inhibitory activity. This could be due to the interaction of the amino acid residue, Ser[1], from the neighboring monomer. On the other hand, micafungin remained bound in the catalytic pocket of the monomeric from of 3CLpro throughout the period of simulation. The list of drugs that are reported in this study provides a rationale to prioritize these potential drugs to be tested preclinically followed by clinical studies to target the SARS-Cov-2 pathogenesis.

Methods

Reagents and drugs. Molecular biology grade DMSO was purchased from Sigma Aldrich (St. Louis, MO, USA), Sterile PBS was from ThermoFisher Scientific (Waltham, MA, USA). 3CLpro inhibitor screening enzymatic assay kits (Catalog #79955-1) were from BPS Biosciences (San Diego, CA, USA). Amprenavir, Atazanavir sulfate, Candicin, Chloroquine Phosphate, Hydroychloroquine Sulfate, and Lopinavir were purchased from Sigma Aldrich (Saint Louis, MO). Beclabuvir, Temsavir were from Medchem Express (Monmouth Junction, NJ). Abacavir (sulfate), Arbidol hydrochloride, Asunaprevir, Atrovastatin, Boceprevir, Daclatasvir, Danoprevir, Darunavir, Delavirdine (mesylate), Edoxudine, Elbasvir, Elvitegravir, Etravirine, Favipiravir, Fumagillin, Glecaprevir, Grazoprevir, Indinavir sulfate, Itraconazole, Ivermectin, Ledipasvir (G-5885), Maraviroc, Methylprednisolone, micafungin sodium, ombitasvir, Oseltamivir phosphate, Paritaprevir, Peramivir, Pibrentasvir, Pimodivir, Pleconaril, Posaconazole, Quinine, Raltegravir (potassium salt), Remdesivir, Ribavirin 5'-monophosphate (lithium salt), Ribostamycin sulfate, Rilpivirine, Saquinavir, Telaprevir, Tenofovir diphosphate (sodium salt), and Velpatasvir were purchased from Cayman Chemicals (Ann Arbor, MI).

Preparation of working solution of ligands. One mg of drug was used to prepare 8 mM stock solution using either DMSO or PBS as solvent. This stock solution was used to prepare the working solution of 250 μM and 500 μM of drugs in PBS.

3CLpro protein preparation. The crystal structure of COVID-19 main protease was retrieved from the protein data bank (www.rcsb.org) with PDB format (ID: 6LU7). Any structural issues if present in the protein were corrected using QuickPrep option in MOE. The QuickPrep option performs a protonation and calculates the minimum energy conformation of the protein. Default parameters of MOE software was used for QuickPrep function.

Preparation of ligands. The drug list of 3987 FDA approved drug molecules and active ingredients were downloaded from SUPERDRUGS2 database in sdf format. The database of these 3987 drugs were imported into Molecular Operating Environment MOE and were cleaned using wash function in MOE. This function rebalances protonation states and regenerate 3D coordinates to their minimum energy conformations. Default parameters of MOE software was used for wash function.

Protein:drug docking studies. Integrated Computer-Aided Molecular design computing method Molecular Operating Environment (MOE) software was used to dock the drugs with 3CLpro protein. Briefly, docking was performed on the ligand site of 6LU7 protease using washed dataset of 3987 drugs. 30 poses for London dG and 20 poses of GBVI/WSA dG were used for final docking. All docking results were sorted by the binding energy using S Score function and they were viewed for accuracy using the ligand interaction function in MOE. The drugs with a binding score (S-Score) of ≤−6.5 were considered for further studies. The

FOI 26-3132 - Document 5

list was further narrowed down based on the listed criteria (1) All peptidomimetic drugs were eliminated as they tend to elicit immune response in the body; (2) Drugs used as immunosuppressants or for the treatment of cardiovascular disorders were eliminated as they tend to be in high risk categories; (3) All prodrugs were eliminated unless the active form was readily available as our in vitro assay is specific for SARS-COV-2 3CLpro and does not provide an option to activate drugs in vitro; and (4) Drugs with a shorter half-life of <30 min were also eliminated. In addition, we also ensured that small molecules which are part of any current Phase I, II, or III clinical trials were included in this study irrespective of their S-Score. For e.g favipiravir has an S score of −4.23 and since it is under clinical trials for COVID-19, we have included favipiravir in the study. This screening led to the database of 56 drugs, which were selected to study their inhibitory effect on SARS-CoV-2 specific 3CLpro enzyme using an in vitro 3CLpro enzymatic assay.

3CLpro enzymatic assay. SARS-CoV-2 specific 3CLpro assay kits were purchased from BPS Biosciences (CA) and enzymatic assay was carried out as per the manufacturer's protocol using 96 well plates. Briefly, 4 ng 3CLpro-MBP tagged enzyme in 30 µl of assay buffer was pre-incubated with 10 µl of (250 µM) drugs for 1 h. The enzymatic reaction was initiated by adding 10 µl (250 µM) fluorescent substrate. The final volume of the assay samples was 50 µl. The final concentration of drugs and substrate in the reaction mixture was 50 µM. Incubation was continued at room temperature for 16–18 h. Fluorescence reading was taken at 360/40 excitation and 460/40 nm emission using Synergy HT fluorescent plate reader. For IC_{50} calculation, drugs were screened from 0 to 100 µM dose range. Wells with 1% DMSO with 4 ng of enzyme and 50 µM of substrate served as positive control with no enzyme inhibition. Wells with 50 µM of GC367 compound (provided by the BPS Biosciences) served as standard inhibitor and negative control. Wells with 1% DMSO with 50 µM of substrate without enzyme served as blank. All the values were subtracted from blank values.

Molecular dynamic simulation studies. The structure of COVID-19 main protease 6LU7 (PDB ID: 6LU7) was prepared in its monomeric and functional dimeric form using the QuickPrep application of MOE2019 with default parameters. This atomistic model was used for generating the input files for all MD simulations.

The best dock pose of micafungin was placed into the binding pocket of the prepared monomeric 3CLprotease structure. The best pose of ivermectin was placed into the monomeric and dimeric protease structure to test which form can stabilize the inhibitor. The simulation cell and NAMD 2.14[39] input files were generated using MOE2019. The crystallographic water molecules were removed prior to solvation. Next, the protein/ligand complexes were embedded in a TIP3P water box with cubic periodic boundary conditions, keeping a distance of 10 Å between the boundaries and the protein. The net charge of the protein was neutralized with 100 mM NaCl. For energy minimization and MD simulations, the AMBER10:EHT force field was used and the electrostatic interactions were evaluated by the particle-mesh Ewald method. Each system was energy-minimized for 5000 steps using the Steepest Descent and Conjugate Gradient method. For equilibration the system was subjected to a 100-ps simulation to gradually heating the system from 10 K to 300 K. Next, a 100-ps NVT ensemble was generated at 300 K followed by an NPT ensemble for 200 ps at 300 K and 1 bar. Then, for each complex, a 100-ns production trajectory was generated for further analysis. The trajectory analysis was done using scripts shared by the CCG support group.

Identification of ligand-binding mode. The protein ligand interaction fingerprint application in MOE2019 was used to study the average binding mode of each inhibitor bound to the monomer or dimer of 6LU7. The calculation used the default parameters on the recorded MD trajectories.

Statistics and reproducibility. One-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison post-hoc test was performed with 99.9% confidence intervals to compare the statistical significance and represented as the mean ± SEM. P values < 0.001 considered statistically significant. Non-linear regression (curve fit) with four variable dose vs inhibition was used to calculate the IC_{50} values. Statistical analysis was performed using GraphPad Prism (version 6.07; La Jolla, CA, USA). All the experiments were carried out minimum three times with triplicates for reproducibility and the representative of three individual experiments is presented in this report. The data generated at different time points were combined to make the final graphs. Investigators performing the assay were blinded for the drugs being tested in the assay.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Supplementary Data 1 provides the data set for Figs. 2–5, and Supplementary Figure 2 and 3. Supplementary Data 2 and 3 provides data set for 3CLpro-OTDs docking study and Supplementary Data 4 for PIs and VNIs (S score for Table 1 and structural analysis for Fig. 6). Supplementary Data 5 provides data set for MD simulation study of 3CLPro homodimer with ivermectin, Supplementary Data 6 for 3CLPro monomer with ivermectin and Supplementary Data 7 for 3CLPro monomer with micafungin (Fig. 7). Supplementary Data 5–7 provides data set for Supplementary Fig. 3 (S score comparison from MD simulation study). Any remaining information can be obtained from the corresponding author upon reasonable request.

Received: 7 August 2020; Accepted: 8 December 2020;
Published online: 20 January 2021

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FOI 26-3132 - Document 5

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Acknowledgements

We thank Travis Field, Tu Dang, Handong Ma, and Yan Wu, Division of Research, PCOM for their technical support during the study. We also thank Dr. Deepa Machiah, Emory University, Atlanta for critical inputs and proof reading the manuscript. This work was supported by Chief Scientific officer funding from PCOM to V.M. and S.T., and partly by National Institute of Allergy and Infectious Diseases (1R03 AI128254-01A1) to S.T.

Author contributions

V.M. and S.T. designed, performed and wrote the manuscript, S.R. performed the experiment and proofread the manuscript. M.E. performed MD simulation studies. G.M.F. helped with molecular docking studies. J.H., S.W., A.M., and L.L. collected the data on drugs. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s42003-020-01577-x.

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Ivermectin as an adjunct treatment for hospitalized adult COVID-19 patients: A randomized multi-center clinical trial

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Research Article

Keywords: SARS-CoV-2, Ivermectin, Randomized controlled trial, Dose-response relationship

DOI: https://doi.org/10.21203/rs.3.rs-109670/v1

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Abstract

Background: It appears that ivermectin can potentially act against COVID-19 infection. Today, it is an urgent need to evaluate the efficacy and safety of ivermectin. The effect of ivermectin therapy on mild to severe COVID-19 patients was investigated.

Methods: A 45-days randomized, double-blind, placebo-controlled, multicenter, phase 2 clinical trial was designed at five hospitals. A total number of 180 mild to severe hospitalized patients with confirmed PCR and chest image tests were enrolled. The radiographic findings, hospitalization and low O_2 saturation duration, and clinical outcomes such as mortality and variables of blood samples were analyzed using standard statistical analyses in SPSS (V20).

Results: Average age of the participants was 56 years (45-67) and 50% were women. The primary and secondary results showed significant changes between day zero and day five of admission (Δ 0/5) in terms of ΔALC5/0, ΔPLT5/0, ΔESR5/0, ΔCRP5/0, duration of low O_2 saturation, and duration of hospitalization (CI = 95%). Risk of mortality was also decreased significantly in the study groups.

Conclusion: Ivermectin as an adjunct reduced the rate of mortality, low O_2 duration, and duration of hospitalization in adult COVID 19 patients. The improvement of other clinical parameters showed that the ivermectin, with a wide margin of safety, had a high therapeutic effect on COVID-19.

Trial Registration: This trial was registered with the Iranian Registry of Clinical Trials website (registration ID IRCT20200408046987N1).

Introduction

The COVID-19 infection has become a pandemic since its first outbreak in Wuhan, China in 2019. This infection is caused by the novel COVID-19 and has created a difficult condition around the world. Thus, there is an important and urgent need to find proper treatments for an effective cure, decrease the virus carriage duration, and thus limit its transmission in society [1-3]. So far, different drugs such as Hydroxychloroquine (HCQ) [4,5], Azithromycin [6], and combination of them have been used against COVID-19. Several studies have demonstrated that chloroquine phosphate and chloroquine sulfate inhibit COVID-19 *in-vitro* [7-9]. In addition, Gautret et al. evaluated a combination of HCQ and azithromycin on COVID-19 infected cells *in-vitro*, and concluded that there was a considerable synergetic effect when the combination was used at doses which imitates the concentrations likely to be taken in humans [8]. Chloroquine, illustrated positive results in Chinese COVID-19 patients in clinical trials by alleviating fever, delaying disease progression, and management of COVID-19 disease (500mg twice per day for ten days) [8,10,11]. However, among the candidate treatments, only three main drugs including Remdesivir, Oseltamivir, and HCQ have been tested in large comparative studies [8,10-15]. Some of studies have shown that Lopinavir, Ritonavir, and Remdesivir have no clear influence on COVID-19 infection and they are associated with many adverse effects [13,16,17]. In addition, HCQ has demonstrated its efficacy in

reducing viral shedding durability [11], and improving patients' clinical status in observational or randomized clinical trials [12,18,19]. Ivermectin is an unexpensive FDA-approved anti-parasitic drug, known to have a wide-spectrum antiviral activity against a variety of viruses in *in-vitro* conditions. It inhibits the proliferation of COVID-19 in cell culture as well [6,20,21]. Moreover, ivermectin shows antiviral activity against some RNA viruses including Zika, dengue, yellow fever, West Nile, Hendra, Newcastle, Venezuelan equine encephalitis, chikungunya, Semliki Forest, Sindbis, Avian influenza A, Porcine Reproductive and Respiratory Syndrome, and HIV-1. Furthermore, there are some studies showing the antiviral effects of ivermectin against DNA viruses such as Equine herpes type 1, BK polyomavirus, pseudorabies, porcine circovirus 2, and bovine herpesvirus 1 [22]. Ivermectin may also act on the COVID-19 virus through three proposed mechanisms that result in reducing the load of the virus. These mechanisms includes: inhibition of nuclear import of host [23], interfering with the attachment of the spike to the human cell membrane [24,25], and inhibition of helicase activity [26]. Based on elementary data from Caly et al., Monash University in Australia, a strategy was designed including early PCR test or chest image to detect positive patients and adding ivermectin to the treatment regimen at different doses, to investigate its possible treatment efficacy on COVID-19 patient.

Materials And Methods

Study design

A randomized, double-blind, placebo-controlled, multicenter, phase 2 study was carried out to examine the effectiveness of oral ivermectin in hospitalized adults (age >18 years) with COVID-19. The trial was conducted at five hospitals (Velayat, Bu Ali, Taleghani, Razi, and Sina) in Qazvin and Khuzestan provinces of Iran. Ethical approval was in accordance with the ethical standards of the Helsinki Declaration (1964, amendment of 2008). The study protocol was approved by the medical ethics committee of Qazvin University of Medical Sciences (registration ID IR.QUMS.REC.1399.017).

Patient selection

Eligible patients with COVID-19 who met the following criteria were admitted: a) Age >18 years; b) signed the informed consent; c) clinical symptoms of suggestive of COVID-19 pneumonia: cough (with or without sputum), fever, pleuritic chest pain or dyspnea; d) mild to severe COVID-19 disease confirmed by chest computed tomography (CT) scan findings compatible with COVID-19 or positive real-time reverse transcription polymerase chain reaction (RT-PCR). Exclusion criteria included presence of severe immunosuppression (e.g., use of immune-suppressants and HIV positive), pregnant women, chronic kidney disease, malignancy, and indications that the patients were unable and/or unlikely to comprehend and/or follow the protocol. The primary endpoint of this trial was clinical recovery within 45 days of enrolment. Clinical recovery was defined as normal fever, respiratory rate, and oxygen saturation (>94) without oxygen therapy sustained for 24h. The patients would be discharged if this trend continued. During the process the criteria for discharge was changed over the course of study. Initially, patients with two successive negative nasopharyngeal samples based on PCR assay (CT value≥35) were separated.

The study sample size was estimated based on a test of equivalence[27]. Using a two-sided test level of 0.05 and a desired statistical power of 90% and under the assumption that each treatment arm would yield a 75 % success rate, the number of patients in the study was obtained equal to 163 patients. Assuming an availability rate of 35%, 30 patients were enrolled per treatment arm.

Interventions

The participants were randomly allocated to six arms including common regimen based on Iran health ministry (Hydroxychloroquine 200mg/kg twice per day), placebo plus common regime, single dose ivermectin (200mcg/Kg, 1 pill per day), three low interval doses of ivermectin (200, 200, 200 mcg/Kg , 3 pills in 1, 3 and 5 interval days), single dose ivermectin (400mcg/Kg, 2 pills per day), and three high interval doses of ivermectin (400, 200, 200 mcg/Kg, 4 pills in 1, 3 and 5 interval days).

Randomization and masking

Eligible patients were randomly allocated to either the standard, Placebo and the ivermectin arms. Randomization according to the severity of the disease was as follows: mild, moderate, and sever. The transposed block randomization sequence, including stratification, was prepared by a statistician not involved in the trial using Random Allocation Software. The patients in six treatment arms enrolment were randomized after calling the central randomization telephone number and receiving randomization information and confirmation. Each patient received the unique patient numbers that were to be used on all study medication containers, case report forms, and to identify all specimens. Pharmacia generated the randomization list and provided the list to the central randomization service.

Laboratory and radiographic tests

The patients systematically underwent an unenhanced chest low-dose computed tomography (LDCT) at reception or soon after, using a single CT machine (16 slice Model Neusof, China). All images were analyzed by expert chest radiologists and classified as compatible or not compatible with pneumonia. Images were considered as compatible with circumferential multifocal ground-glass opacities, vesicular consolidation, or crazy paving template. Novel Coronavirus RNA was recognized by real-time reverse transcription PCR [28]. Negative results for viral RNA detection were defined as a cycle threshold (CT) value≥35.

Treatment

All patients were treated according to “Iranian guideline of hospitalized COVID-19 patients’ management (version 5)”. This comprised oral hydroxychloroquine (HCQ) 200mg/kg twice per day as standard regimen and a heparin prophylaxis in combination with supplemental oxygen. Tablet of ivermectin (14 mg) and placebo were formulated in Alborz Darou pharmaceutical Co., Tehran, Iran. The Participants received drug after signing the consent letter. For all arms, radiographic findings, hospitalization time, clinical outcomes such as mortality, and clinical parameters such as account oxygen saturation testing

and blood sampling absolute lymphocyte count (ALC), C-reactive protein (CRP), white blood cells (WBC), lactate dehydrogenase (LDH), thrombocyte count (PLT), erythrocyte sedimentation rate (ESR), blood urea nitrogen (BUN), and creatinine (Cr) were examined on days zero and five. Assessment of gas exchange requires knowledge of fractional inspired oxygen tension (FiO_2); unless the patient is breathing room air. Hence, all peripheral capillary oxygen saturation was measured in breathing room air at rest.

Statistical methods

The SPSS version 20 (SPSS®, Armonk, NY, USA) software was used for statistical analyses. Shapiro-Wilk test was used to evaluate normality of numerical variables. The quantitative variables were expressed as mean ± SD (standard deviation) or Median ± IQR (Inter Quartile Range). The difference values for each quantitative variable on days zero and five (Δ 0/5) were calculated to check recovery process of patients between groups. Pair T-test / Wilcoxon signed-rank test were used on inter-group comparison. The analysis of variance (ANOVA) was used to compare the mean scores of the groups. To compare the proportions, Pearson chi-square or Fisher's exact test was used. Analyses were based on non-missing data. P-value less than 0.05 was considered statistically significant.

Results

A clinical trial was started to determine the efficient dose of ivermectin for 45 days. A total of 180 patients with mild to severe infection, with positive PCR and chest image tests, hospitalized at Qazvin and Khuzestan provinces hospitals (five hospitals) were selected. The hospitalization term was from 1st of June to 15th of July 2020 (45 days) (Fig 1). Demographics (sex, age), Obesity, Severity on CT, PCR, Respiratory Rate (RR/min), Fever **(°C)**, Systolic and Diastolic Blood pressure (Bp;mmHg), Pulmonary Rate (PR /min) and Oxygen saturation (%) of the patients are reported in Table 1. The majority of patients had favorable outcomes and Mann-Whitney U Test and χ^2 test were used to analyze and compare the variables at the day one and five of admission (Δ 0/5). As reported in Table 2, complete blood count (CBC) evaluation of the patients among the standard and placebo and ivermectin treated (arms 3 to 6) showed that ivermectin had a good effect on blood biomarkers and improved other clinical parameters such as ALC, CRP, PLT , ESR , LDH, BUN, and Cr.

Patient care:

The majority of patients had desired consequence and were discharged from the hospitals. The results of preclinical consequences in Table 3 indicate a reduction in mortality rate in patients receiving ivermectin treatment to 0, 10, 0 and 3.3% for arms 1- 4 respectively, compared to the standard and placebo plus standard arms which was 16.7% and 20% respectively. Moreover, the decrease in hospitalization and low O_2 saturating terms was significant in ivermectin treated 1-4 arms compared to the two untreated controls (P=0.006 and P=0.025 respectively). The lowest mortality rate (0%), hospitalization duration (5days), and duration of low O_2 saturatin (2days) was observed arm 3 with single dose of 400mcg/kg

ivermectin. Table 4 lists the risk of mortality for comparison between ivermectin treated and untreated groups with CI: 95%. The estimation confirmed 15% reduction of the mortality with risk ratio of 0.18.

Discussion

Totally, 180 patients infected by COVID-19 virus were allocated to six arms and received single or interval doses of ivermectin. The positive effect of ivermectin therapy in patients receiving this drug was a reduction in mortality rate compared to the standard (16.7%) and placebo (20%) arms. The improvement of clinical manifestations of the patients was observed in all ivermectin groups in comparison with standard and placebo arms. From the beginning of the outbreak, various antiviral drugs such as Acyclovir, Ganciclovir, Oseltamivir and ribavirin have been tested. Unfortunately however, these drugs have not been effective in curing or alleviating the symptoms of COVID-19 [29]. Studies have shown that among the FDA-approved antiviral drugs, like ribavirin, Penciclovir, Nitazoxanide, Nafamostat and Remdesivir, the last two drugs are highly effective against novel coronavirus infection *in-vitro*[30]. Recently, ivermectin, another FDA-approved drug, was reported by Caly et al. to inhibit the *in-vitro* replication of novel Coronavirus. The results of this study showed that a single treatment of ivermectin was able to induce a 5000-folds reduction in the viral RNA 48hrs in cell culture[6]. As illustrated in Figure 2, the main *in vitro* proposed mechanism of activity of ivermectin hinders the viral load through inhibition of the interaction of importin α and β_1 proteins. Recently, a clinical trial reported lower mortality rates in hospitalized patients with COVID-19 who received ivermectin therapy [31]. Our clinical trial was designed with features such as double-blind, placebo-controlled, multicenter, open-label, phase 2/3, and randomized controlled trial. It is suggested by previous studies that the initial disease intensity assessment cannot rely only on clinical examination[11]. In this clinical trial, single and multiple oral doses (200mcg/Kg and 400 mcg/Kg) of ivermectin were added to the standard regimen of patients (treatment protocols of Iranian health ministry for COVID-19). At the time of writing the manuscript, the HCQ is stopped by world health organization (WHO) from all trials. As illustrated in Tables 2 and 3, the cure effect of ivermectin in different doses was confirmed by both laboratory and preclinical parameters. The results of ivermectin treated subgroups with the standard and placebo groups showed a difference between each subgroup and s,p groups. The combined effects of different-doses of ivermectin on patients' medication regimens shortened the duration hospitalization and low O_2 saturation. Promotion of other clinical parameters such as ALC, CRP, PLT, ESR, LDH, BUN and Cr showed that the ivermectin with a wide margin of safety had a higher therapeutic index and efficacy against COVID-19. One of the limitations of ivermectin in clinical utility is its potential to cause toxicity. Studies have shown that this defect can be eliminated by changing the formulation and pharmacokinetic properties. Therefore, a systematic design based on concentration of ivermectin is essential [32]. Schmith et al. showed in a study based on the pharmacokinetic simulations that ivermectin may have limited therapeutic utility on control COVID- 19. The reason is that the concentration of inhibitor required to act on the COVID-19 virus is much higher than the maximum plasma concentration by managing the approved dose [20,33]. Based on this limitation, they proposed ivermectin inhalation therapy; however, didn’t consider the host immune

response in human. In addition, Caly et al. did not clarify if lower doses were effective or not. However, the antiviral properties of ivermectin have been demonstrated by targeting critical cellular process of the mammalian cell. Therefore, in line with our study, it is speculated that ivermectin administration, even at low doses, can reduce the loading of the virus in the early stages [34]. In addition to above explanations, sustainability of ivermectin in different tissues of animals and humans has been reported by various studies [35-37]. As mentioned in the introduction, it seems that the mechanisms of antiviral activity of ivermectin against various viruses is mediated via targeting importin α/β-mediated nuclear transport of HIV-1 integrase and NS5 polymerase; NS3 helicase; nuclear import of UL42; and nuclear localization signal-mediated nuclear import of Cap [38-41]. Although, at the time of the writing of this paper the HCQ was eliminated from the clinical care protocols of our clinical centers, we did the dose finding of ivermectin based on the standard care (HCQ) of Iranian ministry protocols at start. Based on findings, combined designs of ivermectin with other antiviral drugs appeared to have desirable results. In an unpublished study, the combination of ivermectin with HCQ was investigated [42]. As to the safety of using the high doses of ivermectin through this study, the flow-ups showed that there were no side effects such as nausea or skin rash during the trial. In a three arms phase 3 clinical trial, ivermectin was administered in two doses of 200 and 400 mcg/kg in patients with dengue fever. The main purpose of this trial was to evaluate safety of ivermectin in children and adult patients and the results showed that one daily dose of ivermectin treatment for three days was safe [43]. There have been several reports about drug treatments that have significantly affected viral load in COVID-19 patients. The novel coronavirus has several evolutionary phases, so that in the first clinical phase, including lower respiratory tract infection (LRTI) and upper respiratory tract infection (URTI) symptoms are associated with a high viral load and the incidence of early lung lesions on LDCT [44,45]. Here the focus of this study was to emphasize on the proper dose of ivermectin (single dose of 400mcg/Kg) that may reduce the viral load and affect both LRTI and URTI symptoms. However, for more efficacy of drug especially on URTI symptoms further studies are needed to experiment different combinations of immunological triggering agents such as corticosteroids on the mild to severe patients. Moreover, because of the direct impact of ivermectin on virus and as mentioned above and its sustainability in different organs specially the pulmonary tissue, further researched should be implemented to investigate the possible prophylactic effect of this drug against the first entrance of virus or after observation LRTI symptoms. Moreover, as listed in Tables 3 and 4, the lowest mortality rate was observed in arms 1-4 in comparison with 16.7% and 20% rates in the standard and placebo plus standard arms respectively. The risk base estimation with CI: 95% in Table 4 confirm 15% reduction of mortality or about 5.5 folds increase of the mortality rate in control groups. These results are in line with Rajter et al. [31] who reported a 40% drop in mortality of critical COVID-19 patients after oral administration of ivermectin at least in one dose (200mcg/Kg).

Limitations

The sample size was not large and the study was limited to the selected hospitals. Studies in areas with a maximum prevalence of COVID 19 and on patients with more diverse conditions such as body mass index over 40, different underlying diseases, or younger patients are needed. Without these limitations, a

stronger trial study could be carried out. Ongoing studies with larger sample sizes, using strategies to enhance the antiviral potency of ivermectin and its combination with other antivirals or higher-dose regimens, and focus on severe COVID-19 cases are recommended.

Declarations

Acknowledgements

The supports of deputy of research and technology of Qazvin University of Medical Sciences and Qazvin Science and Technology Park are appreciated. We thank Cellular and Molecular Research Center and Infection Prevention Center of Qazvin University of Medical Sciences and all the clinical, technical, and paramedical staff of the wards and laboratories for their support and cooperative attitudes.

Funding: The present study is financially supported by the research deputy of Qazvin University of Medical Sciences and Science and Technology Park, Qazvin, Iran.

Potential conflicts of interest: No reported conflicts of interests. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Data Sharing Statement: The authors confirm that the data supporting the findings of this study are available in a supplementary materials file (not provided for this preprint).

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Tables

Table1: Baseline characteristics and demographic evaluation of patients enrolled in clinical trial

Median [interquartile range], n (%), S: common regimen, P: placebo plus common regime group, Arm1: Single dose ivermectin (200 mcg/Kg), Arm2: three dose ivermectin (200 mcg/Kg), Arm3: single dose ivermectin (400 mcg/Kg), Arm 4: three dose ivermectin (400, 200, 200 mcg/Kg).

Table2: Baseline characteristics and Complete blood Count (CBC) evaluation of patients enrolled in clinical trial.

median [interquartile range], n (%), S: common regimen , P: placebo plus common regime group, Arm1: Single dose ivermectin (200 mcg/Kg), Arm2: three dose ivermectin (200 mcg/Kg), Arm3: single dose ivermectin (400 mcg/Kg), Arm 4: three dose ivermectin (400, 200, 200 mcg/Kg). a. Mann-Whitney U Test, b. χ^2 test, Δ 0/5= Variable in 5th- Variable in admission day.

Table 3: Clinical Consequences occur in different arms of the study

		Group						
		Control groups (S +P)		Ivermectin Groups				
		S	P	Arm1	Arm2	Arm3	Arm4	Total
Sex	male	16 (53.3)	14 (46.7)	12 (40.0)	19 (63.3)	16 (53.3)	13 (43.3)	90 (50.0)
	female	14 (46.7)	16 (53.3)	18 (60.0)	11 (36.7)	14 (46.7)	17 (56.7)	90 (50.0)
Age (year)		55 [45 - 70]	58 [45 - 68]	61 [42 - 68]	53 [42 - 65]	54 [47 - 60]	54 [46 - 65]	56 [45 - 67]
BMI (Kg/m2)		26.0 [24.1 -27.6]	25 .6 [23.9 -26.9]	26.1 [24.8 - 28.0]	26.4 [25.5 - 27.2]	27.7 [25.7 -32.6]	25.1 [23.9 -26.2]	26.0 [24.7 -27.4]
Severity on CT	negative	0 (0)	0 (0)	0 (0)	2 (6.7)	0 (0)	0 (0)	2 (1.1)
	mild	4 (13.3)	5 (16.7)	8 (26.7)	2 (6.7)	4 (13.3)	2 (6.7)	25 (13.9)
	mode	23 (76.7)	23 (76.7)	21 (70.0)	20 (66.7)	21 (70.0)	23 (76.7)	131 (72.8)
	sever	3 (10.0)	2 (6.7)	1 (3.3)	6 (20.0)	5 (16.7)	5 (16.7)	22 (12.2)
PCR	positive	18 (60.0)	14 (46.7)	23 (76.7)	23 (76.7)	29 (96.7)	21 (70.0)	128 (71.1)
	negative	12 (40.0)	16 (53.3)	7 (23.3)	7 (23.3)	1 (3.3)	9 (30.0)	52 (28.9)
RR (/ min)		28 [25 - 29]	28 [27 - 34]	28 [26 - 29]	30 [28 - 34]	22 [20 - 24]	29 [28 - 30]	28 [25 - 30]
Fever (°C)		36.8 [36.7 - 37.0]	36.8 [36.8 - 37.2]	36.8 [36.4 - 37.1]	36.8 [36.7 -37.2]	36.5 [36.5 - 36.8]	37.0 [36 - 37.2]	36.8 [36.5 - 37.1]
Systolic BP (mmHg)		110 [110 - 125]	112 [110 -135]	125 [110 -125]	110 [110 -125]	120 [110 - 120]	140 [120 - 160]	115 [110 -125]
Diastolic BP (mmHg)		80 [80 - 80]	80 [80 - 80]	80 [80 - 80]	80 [80 - 80]	75 [55 - 80]	80 [70 - 90]	80 [80 - 80]
PR (/min)		80 [75 - 85]	80 [72 - 85]	78 [75 - 85]	80 [78 - 95]	85 [80 - 93]	85 [78 -100]	80 [76 - 90]
O2 Sat (%)		89 [85 - 91]	88 [85 - 90]	90 [88 - 92]	88 [85 - 90]	91 [87 - 94]	89 [82 - 90]	89 [85 - 91]

	Group						
	Control groups (S +P)		Ivermectin Groups				
	S	P	Arm1	Arm2	Arm3	Arm4	Total
WBC (per µl)	8300 [5000 – 12000]	6600 [4600 - 400]	5750 [4400 – 7900]	5150 [4400 - 9200]	6300 [4800 - 7800]	5200 [4400 - 6300]	6500 [4500 – 8850]
ΔWBC5/0	-300[-2500-800]	-350[-1200-900]	-200[1600-300]	150[-900-1000]	0[-1000-300]	650[-300-1800]	0 [-150-900]
p-value							0.217[a]
ALC (per µl)	1275 [770 - 1704]	1135 [783 1575]	1017.50 [792 1443]	1165.50 [816 - 1620]	1188 [888-1449]	1020 [561 - 600]	1124 [787 - 1584]
ΔALC5/0	78[-310-340]	58[-360-408]	357[5-625]	373[-38-565]	414[89-588]	316[-180-845]	288[-156-549]
p-value							<0.001*
PLT (per µl)	180 [164 - 227]	189 [154 245]	173 [133 - 205]	162 [121 - 217]	215 [175 256]	179 [141 - 237]	178 [149 – 227]
ΔPLT5/0	2[-11-27]	4[-27-28]	40.50[11-70]	39.50[10-74]	13[0-99]	30[8-61]	20[-3-59]
p-value							<0.001*
ESR (mm/h)	32 [13 - 56]	29 [17 - 38]	37 [24 - 50]	39 [25 - 53]	35 [20 - 70]	50 [36 - 59]	36 [20 - 56]
ΔESR5/0	-4.5[-13-1]	-2.5[-6-1]	-12.5[-18- -4]	-7[-16- -2]	-2[12-0]	-6[-11 -2]	-5[-14- -1]
p-value							0.015*
CRP (mg/dl)	28 [14 - 46]	27 [11 - 42]	20 [12 - 44]	39 [20 - 47]	25 [20 - 48]	34 [20 - 54]	29 [16 - 47]
ΔCRP5/0	-4.5[-18-4]	-2.50[-15-4]	-11.50[-29--3]	-19[-30 - -1]	-17[-36- -9]	-17[-29--3]	-11[-26.50 - -1]
p-value							<0.001*
BUN (mg/dl)	19 [14 - 26]	23 [12 - 29]	19 [14 - 26]	14 [12 - 19]	14 [12 – 19]	15 [13 - 20]	17 [12 - 22]
ΔBUN5/0	-2.50[-7-2]	-2[-6-1]	-3.50[-8-1]	-2[-4- -1]	-1.50[-4.50-2]	1.50[-2-4]	-2[-5-2]
p-value							0.109
Cr (mg/dl)	1 [0.8 - 1.2]	[1.1 - [0.9 - 1.5]	1 [0.8 - 1.3]	0.9 [0.8 - 1.1]	0.9 [0.85 -1.05]	0.9 [0.8 - 1.2]	1 [0.8 - 1.2]
ΔCr5/0	-0.05[-0.2-0.1]	-0.1[-0.3-0.1]	-0.10[-0.2-0.1]	-0.05[-0.1-0.1]	0[-0.2-0]	-0.05[-0.2-0.1]	-0.1[-0.2-0.1]
p-value							0.64

Consequence		Group						
		Control groups (S +P)		Ivermectin Groups				
		S	P	Arm1	Arm2	Arm3	Arm4	P value
Tachypnea Off (day)		2 [2 - 3]	3 [2 - 4]	2 [1 - 3]	3 [2 - 4]	3 [3 - 5]	3 [3 - 5]	0.584
Fever Off (day)		0 [0 - 1]	0 [0 - 1]	0 [0 - 1]	0 [0 - 1]	1 [1 - 1]	1 [0 - 2]	0.102
Duration of low O2 Sat		3 [2 - 5]	4 [2 - 6]	2 [1 - 2]	3 [2 - 5]	2 [1 - 4]	5 [3 - 6]	0.025*
Duration on hospital stay (Day)		7 [7 - 9]	8 [6 - 11]	6 [5 - 7]	8 [6 - 9]	5 [4 - 7]	7 [6 - 10]	0.006*
Outcome1	**Alive**	25(83.3)	24 (80)	30 (100)	27 (90)	30(100)	29(96.7)	0.001*[b]
	Death	5 (16.7)	6 (20)	0 (0)	3 (10)	0 (0)	1 (3.3)	

a. Mann-Whitney U Test, b. χ^2 test, Δ 0/5= Variable in 5th- Variable in admission day, **S**: common regimen , **P**: placebo plus common regime group, **Arm1**: Single dose ivermectin (200 mcg/Kg), **Arm2:** three dose ivermectin (200 mcg/Kg), Arm3: single dose ivermectin (400 mcg/Kg), Arm 4: three dose ivermectin (400, 200, 200 mcg/Kg).

Table 4. Risk of mortality in study groups (estimates and 95% Confidence Intervals)

Point Estimates		Confidence Limits
Type	**Value**	**Lower, Upper**
Risk in Ivermectin groups	3.3%	1.0, 8.5
Risk in Control groups	18.3%	10.4, 30.1
Overall Risk	8.3%	5.0, 13.4
Risk Ratio	0.18	0.06, 0.55
Risk Difference	-15%	-25.3, -4.7°
Prevented fraction in Control groups	54.6%	23, 50
Prevented fraction in Ivermectin groups	81.8%	45.3, 94.0

Type: Taylor series, ° 95% confidence limits testing exclusion of 0 or 1, as indicated, P-values < 0.05 and confidence limits excluding null values (0,1, or [n]) are highlighted.

Figures

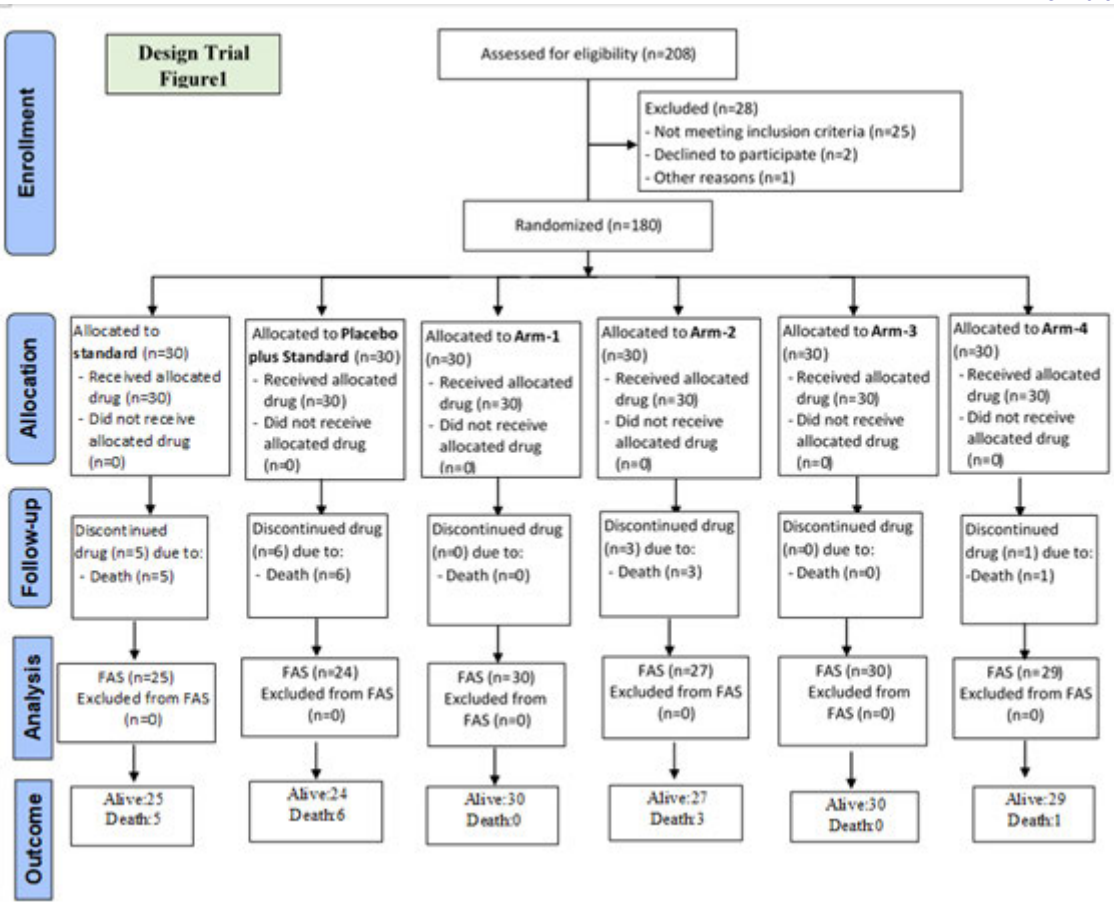


Figure 1

Design Trial

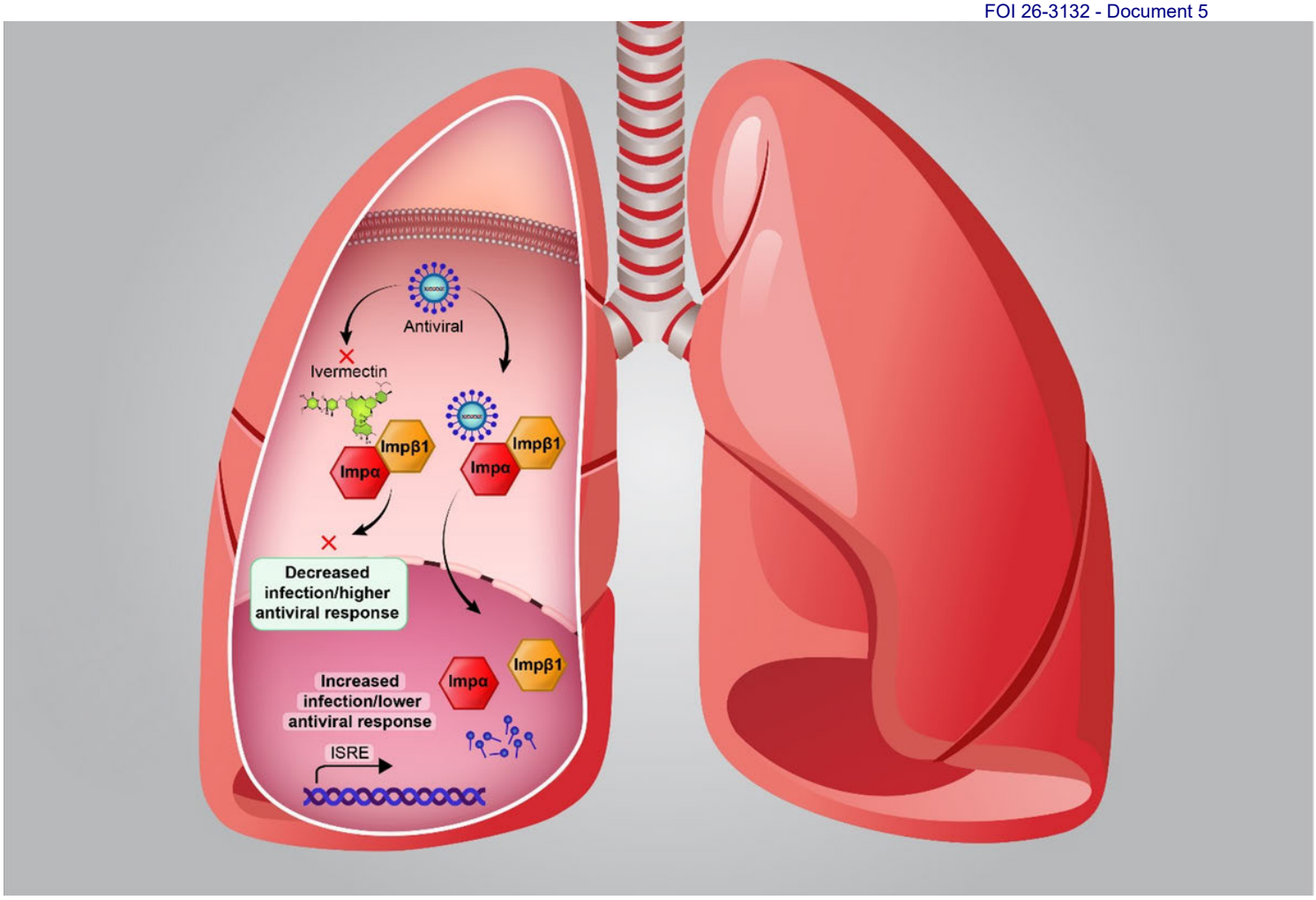


Figure 2

Mechanism of ivermectin inhibition on coronavirus (Designed by the researcher)

FOI 26-3132 - Document 5

Biological Trace Element Research (2021) 199:2882–2892
https://doi.org/10.1007/s12011-020-02437-9

Zinc and COVID-19: Basis of Current Clinical Trials

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Received: 13 August 2020 / Accepted: 11 October 2020 / Published online: 22 October 2020
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Abstract

Coronavirus disease-2019 (COVID-19) pandemic continues to threaten patients, societies, and economic and healthcare systems around the world. Like many other diseases, the host immune system determines the progress of COVID-19 and fatality. Modulation of inflammatory response and cytokine production using immunonutrition is a novel concept that has been applied to other diseases as well. Zinc, one of the anti-inflammatory and antioxidant micronutrient found in food with well-established role in immunity, is currently being used in some clinical trials against COVID-19. This review integrates the contemporary studies of role of zinc in antiviral immunity along with discussing its potential role against COVID-19, and ongoing COVID-19 clinical trials using zinc.

Keywords COVID-19 · Clinical trials · Immunity · Zinc

Introduction

As the whole world is grappling with coronavirus disease-2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), there is a frantic race for finding treatment regimens based on current knowledge until effective vaccine and ad hoc drugs are developed. Indeed, therapeutic approaches against COVID-19, mostly centered on the management of its immunopathology and/or tailored to directly control viral replication, are for now based on off-label prescription. Several human trials are currently in progress to assess the therapeutic indexes of drugs already approved for other diseases (i.e., drug repurposing), and in combination with dietary supplements like vitamin C, vitamin D, vitamin B_{12}, probiotics, and zinc (Zn). Zn is a common theme in both prophylactic and curative COVID-19 clinical studies using nutritional supplements.

Zn, the second most abundant trace metal in the human body after iron, is essential for multiple cellular functions including maintenance of immune health. Notably, "Zn proteome" is estimated to encompass around 3000 proteins [1] including ~ 750 Zn finger transcription factors, a number of which are deeply implicated in homeostasis and inflammation. Ever since Zn deficiency was demonstrated as a human disease more than 56 years ago, it has been implicated in immune dysfunction, growth retardation, hypogonadism, and cognitive impairment [2, 3]. Importantly, Zn also plays a critical role in antiviral immunity [4, 5]. Readers are referred to specialized reviews on essential role of Zn in human health (including Zn homeostasis and transporters) and other non-viral/viral diseases [5–16].

With so many clinical trials going on against the COVID-19 pandemic, there is scientific curiosity about the nature and basis of these trials [17]. In this review, we will discuss the basis of clinical trials using adjunct Zn therapy for COVID-19 through the prism of its indispensable role in antiviral immunity along with briefly discussing immunopathology of COVID-19.

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COVID-19 Immunopathology

As already shown for SARS and Middle East respiratory syndrome (MERS), the host immune response plays a central role also in the pathology associated to SARS-CoV-2 infection, as the fatalities occurring among COVID-19 patients are often preceded by the so-called cytokine storm, a massive systemic release of proinflammatory cytokines such as interleukin (IL)-1b, IL-6, and tumor necrosis factor-α (TNF-α). These events ultimately contribute to acute respiratory distress syndrome (ARDS) and lead to vascular hyperpermeability, diffuse coagulopathy, multi-organ failure, and eventually death [18, 19]. These cytokines are mainly secreted by innate immune cells such as monocytes, macrophages, and dendritic cells (DC), which are supposed to act mainly in the early phases of infections. However, cytokine storm occurs at late stages of SARS-CoV-2 infection, when the adaptive, instead of the innate immune response, is known to become prominent.

A series of timely coordinated actions are required for an effective immune response against viral infections, and the agenda in which these actions move may be outlined by opposing early/innate versus late/adaptive immune response waves. Firstly, innate immune and infected tissue cells initiate primitive and unspecific reactions in an attempt to restrain viral replication by producing type-I interferons (IFN), IFN-α and IFN-β, and to attract blood cells to the site of infection by producing chemokine and TNF-α, which activates endothelial cells enhancing vascular adhesion and diapedesis of leukocytes, and by secreting other proinflammatory cytokines to activate leukocytes. It is peculiar that, while SARS-CoV-2 replication in permissive cells may be effectively inhibited by exogenously added type-I IFN, endogenous production of IFN-α and IFN-β upon infection is inhibited by proteins encoded by SARS-CoV-related viruses, whereas chemokines and inflammatory cytokines are highly produced contributing to an unbalanced response [20, 21].

During this first phase of the immune response, DC migrate to lymph nodes and initiate a complex and slower intercellular cascade, ultimately culminating in the expansion and maturation of adaptive, virus-specific T and B lymphocytes. Seroconversion—i.e., the appearance in the blood of antibodies against immunogenic viral proteins—and probably the clonal expansion of virus-specific T cells, occurs in most COVID-19 patients 10 days after symptom onset [22] and severe disease and cytokine storm are associated in time and magnitude with the immunoglobulin response in SARS-CoV-1 [23] and SARS-CoV-2 infection [24, 25].

Strikingly, a similar cytokine storm is also the most notable side effect of chimeric antigen receptor T (CAR-T) cell therapy, and its severity is forecasted by the extent of tumor burden [26], leading to the hypothesis that a high antigen (i.e., viral) load at the time of the (late) adaptive immune cell activity against SARS-CoV-2, when antibodies and virus-specific T cells arise, may favor a massive second wave of cytokine release by innate cells in a positive feedback loop. Much effort is now trying to decipher, in the immunotherapy setting, the exact mechanisms pushing this positive feedback from adaptive to innate cells, which may deserve our attention to anticipate plausible pathways to interfere with COVID-19 immunopathology. One line of evidence points to cytokines produced by T cells upon antigenic stimulation, namely to TNF-α [27] and granulocyte-macrophage colony-stimulating factor (GM-CSF) [28], as triggers of the secondary systemic toxic cytokine release by innate cells. Importantly, these two cytokines are unnecessary (both) or detrimental (GM-CSF) for on-target effects of CAR-T cells [28], which suggests that they may be dispensable also for the antiviral response to SARS-CoV-2.

There is a clear sex- and age-dependent bias in COVID-19-related mortality. Better outcomes in women, for instance, may be the result of a more pronounced IFN-α production by plasmacytoid DC [29] during initial phases of infection. The more effective viral containment operated by such a response could be assumed to lower the antigen load at the onset of the adaptive wave and prevent the cytokine storm. In studies from patients during the 2003 SARS-CoV-1 outbreak, treatment with synthetic type-I IFN in conjunction with steroids has proven useful to ameliorate pneumonia course [30], and early, in contrast to delayed, type-I IFN treatment rescued mice from death after SARS-CoV-1 infection [31]. On the other hand, aging of the immune system, which is associated to chronic low-level activation of innate cells, also entails a delayed and inflated response upon pathogen encountering [32]. Lastly, with age, adaptive cells become slow in mounting antigen-specific responses to novel pathogens secondary to the loss of the naive B and T cell repertoire due to thymic involution [33].

Immunological Functions of Zn

Data gleaned from seminal animal model studies have added considerable knowledge about the principal roles of Zn in the immune system. Zn is an essential micronutrient, which plays an important role in the physiology of the immune system by acting as signaling molecule. Zn not only acts as an anti-inflammatory agent but also functions as an antioxidant stabilizing membrane [34]. Zn deficiency impinges on survival of immune cells and adversely affects important functions such as phagocytosis, target cell killing, and cytokine production (Fig. 1). It is clearly evident from studies on preclinical models that Zn deficiency plays a role in thymus and lymphoid tissue atrophy [35] and declines in the mechanisms of activation of both helper T cell [36] and cytotoxic $CD8^+$ T cell responses [37]. Specifically, Zn deficiency leads to compromised immune system, as evidenced by degeneration of thymus,

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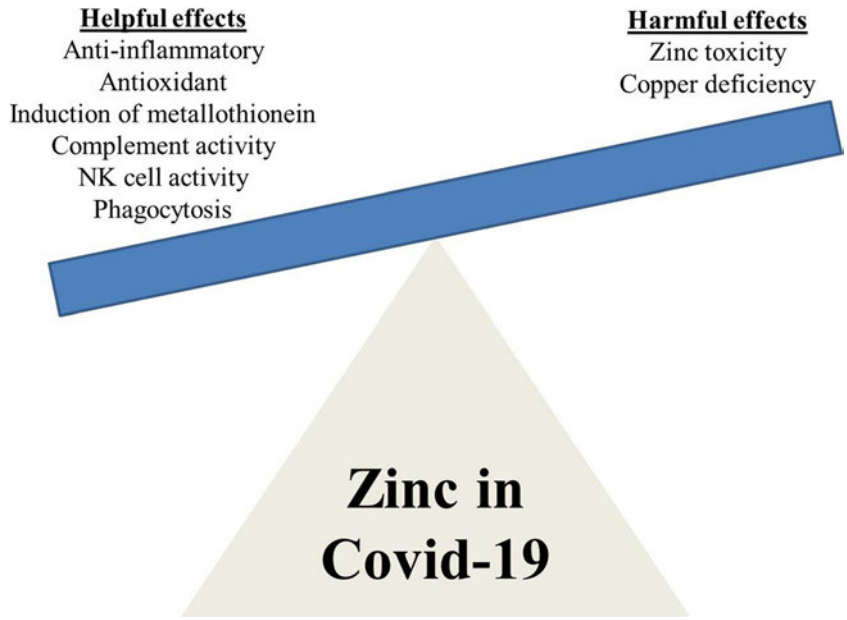


Fig. 1 Balance between helpful and harmful effects of zinc for COVID-19

lymphopenia, and defective lymphocyte responses [3, 5, 34]. Zn deficiency causes immunodeficiency with severe lymphopenia that is characterized in part by a considerable decrease in developing B cell compartments in the bone marrow [3, 38]. Moreover, Zn potentiates IFN-α effect by an order of magnitude [39] which can be used to counteract IFN antagonism by SARS-CoV-2 proteins.

A marked neutrophilia is observed in severe COVID-19 patients [40]. Zn gluconate supplementation is able to reduce airway neutrophil infiltration and TNF-α release by inhibiting NF-kB-dependent transcription of inflammatory genes [41]. The Zn finger CCHC-type containing 3 (ZCCHC3) potentiates TLR3 signaling induced by double-strand RNA [42], which is produced during intracellular replication of coronaviruses.

In vitro, Zn deficiency induced enhanced IL-6 and IL-1β production, as well as higher expression of intercellular adhesion molecule 1 (ICAM-1), important for leukocytes' extravasation, of CD86, a co-stimulatory molecule involved in T cell activation, and of HLA-DR, a MHC-II molecule, in cultured human monocytic cells [43]. Moreover, aged mice have lower plasma Zn concentration, and this was associated to higher IL-6 and ICAM-1 gene expression in splenocytes [43]. Thus, Zn supplementation may be instrumental to reduce inflammatory cytokines, particularly IL-6 and IL-1β, and at the same time to enhance the protective, though inhibited, type-I IFN response in SARS-CoV-2 infection. Zn deficiency indicates towards the decreased interferon-γ (IFN-γ) production, a major component of Th1 cytokine panel [44]. Since Th1 and Th2 responses are mutually inhibited by the effect of their respective signature cytokines IFN-γ and IL-4, such imbalance between Th1 and Th2 might lead to dysfunctions in cell-mediated immune response [34].

There can be a whole lot of factors for the development of Zn deficiency, whether geographic, socioeconomic, nutritional, or as a result of disease pathologies such as chronic viral infections. Notably, during infection, Zn levels decrease significantly and a person's requirement for Zn may increase with the severity of the infection. Aged individuals, infants, and chronic alcoholics are particularly more susceptible to Zn deficiency, increasing their chances of acquiring life-threatening viral infections [45–48]. As Zn is indispensable for a strong immune response, Zn deficiency (persistent hypozincemia [serum Zn < 70 μg/dL]) can considerably dampen innate as well as adaptive antiviral immunity [4, 5].

The Role of Zn in Antiviral Immunity

There is very scant information available on the role and effect of Zn in SARS and coronavirus disease, even though literature is rapidly increasing [17, 49]. Much of the current knowledge about the use of Zn as an antiviral therapy and immunomodulatory agent has originated from studies done with other viral diseases (Readers are referred to these specialized articles for role of Zn in viral diseases [4, 5]). Zn is an essential constituent of thymulin hormone, which is involved in maturation and differentiation of T cells in thymus gland. Zn not only plays an important role in IL-2 and IFN-γ production but also stimulates macrophages to produce IL-12. IL-12 activates the natural killer cells and T cytotoxic cells. Both IFN-γ and IL-12 play a crucial role in destruction of various pathogens. Among anti-inflammatory cytokines, only IL-10 production is affected by Zn deficiency, whose enhanced levels adversely affect the functioning of macrophages and Th1 response [50].

FOI 26-3132 - Document 5

The rationale behind the use of Zn in COVID-19 studies aroused from the observation that Zn ions and Zn ionophores, such as pyrithione (PT), have previously been described as potent inhibitors of various RNA viruses [51]. Also, targeting of RNA-dependent RNA polymerase (RdRps) of RNA viruses like SARS-CoV-2 is more suitable for antiviral drug development as RdRp activity is strictly virus-specific and may be blocked without severely affecting key cellular functions. The basis of Zn therapy in coronavirus infection is shown in Fig. 2. It is known previously that Zn ions are involved in many different cellular processes, proper folding, and activity of various cellular enzymes and transcription factors. On the other hand, Zn^{2+} is probably an important cofactor for numerous viral proteins as well. Zn^{2+} can serve as intracellular second messenger and may trigger apoptosis or a decrease in protein synthesis at elevated concentrations [52–54]. Metallothioneins, a family of metal-binding proteins, regulate homeostasis of Zn and copper, alleviate heavy metal poisoning, and lessen the superoxide stress. Various evidence indicates that metallothionein expression is increased in response to bacterial and viral infections, and it is anticipated that upregulated biosynthesis of human metallothionein might play a significant role in nutritional immunity at the time of active infection [55].

In cell culture studies, inhibition of the replication of various RNA viruses was reported using high Zn^{2+} concentrations and addition of compounds that stimulate cellular import of Zn^{2+}, such as hinokitiol, pyrrolidine dithiocarbamate, and PT as mentioned above (reviewed in) [5]. Around 10 years ago, an in vitro study demonstrated that the Zn ionophore PT in combination with Zn^{2+} is a potent inhibitor of the replication of SARS-CoV-1 [51]. In addition, authors also studied the effect of Zn ions on the RdRp of SARS-CoV-1 and demonstrated that Zn^{2+} directly inhibited the in vitro activity of RdRp (Fig. 2). More specifically, Zn^{2+} was found to block the SARS-CoV-1 RdRp elongation step along with reduced template binding. Further strengthening their claim, authors also showed that the Zn^{2+}-mediated RdRp inhibition of SARS-CoV-1 could be reversed through the addition of a Zn^{2+} chelator [51].

Recently, Finzi (2020) has reported that treatment of four COVID-19 cases with high dose of zinc salts initiated the reduction of disease symptoms within 24 h after initiation of high dose zinc salt lozenges [56]. As adjuvant therapy, Zn (in appropriate dose) may provide protection through decreasing lung inflammation, enhancing mucociliary clearance, inhibiting the ventilator-induced lung injury, and immunomodulation in COVID-19 patients [49].

COVID-19: Interventional Clinical Trials of Drugs/Nutritional Supplements with Zn

As of 4 August 2020, 22 studies were found after searching for Zn and COVID-19 at the clinical trial website https://clinicaltrials.gov/ct2/home. However, upon narrowing down the search criteria, 19 studies were clinical trials and the remaining 3 were observational studies (NCT04326725, NCT04407572, NCT04412746). Furthermore, among the remaining 19 studies, one study (NCT04371952 entitled DYNAMIC Study [DoxycYcliNe AMbulatoIre COVID-19]) was based on the fact that doxycycline, a tetracycline antibiotic, is known to chelate Zn from matrix metalloproteinases, which may help in part to inhibit COVID-19 infection by limiting its ability to replicate in the

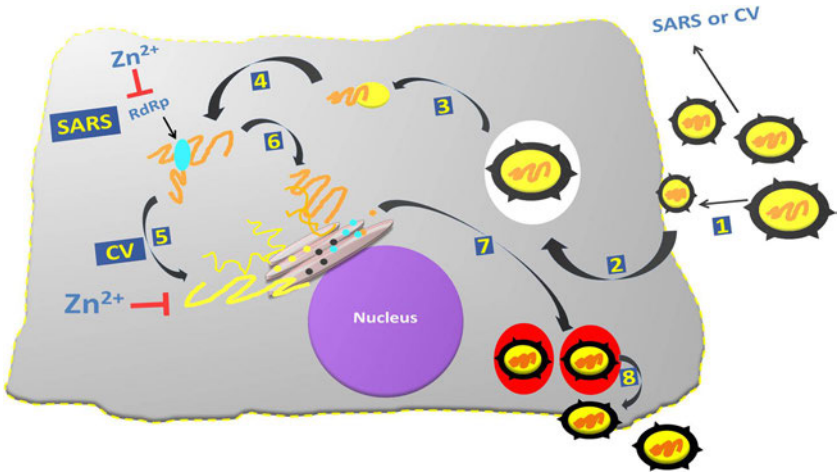


Fig. 2 Schematic representation of different stages of SARS-CoV-1 and coronavirus replication cycle. Viral attachment (1), entry (2), uncoating (3), transcription (4), viral protein translation (5), replication (6), assembly and maturation (7), and finally viral release (8). Data from in vitro studies has demonstrated two mechanisms by which zinc interferes with these viruses' replication cycle steps that include viral genome transcription for SARS-Cov-1 (5), and viral protein translation and polypeptide processing for CV (6). However, to date, no studies have shown zinc-mediated inhibition of SARS-CoV-1 or CV entry, receptor binding, uncoating, assembly, and/or viral particle release. CV, coronavirus; RdRp, RNA-dependent RNA polymerase; SARS, severe acute respiratory syndrome coronavirus; Zn, zinc

host. There is no intervention of Zn supplementation to the patient, so this study was ruled out for present review. Three more studies (NCT04485130, NCT04491994, NCT04485169) also did not use zinc in the experimental arm, so similarly left out from the present article.

Among the remaining 15 studies (Table 1), 4 studies are exclusively using different combinations of dietary supplement including Zn as an intervention against COVID-19 (NCT04351490, NCT04342728, NCT04323228, NCT04468139 at serial nos. 1, 2, 3, and 4 of Table 1, respectively). None of the four studies is completed yet at the time of writing this review. The NCT04351490 trial is using combination of vitamin D_3 with Zn gluconate with the rationale that these two micronutrients are able to modulate the immune response by reducing the inflammatory storm. Vitamin D, a steroid hormone synthesized from cholesterol, has recently been reported to have anti-fibrotic, anti-inflammatory, and immunomodulatory properties on the basis of various studies done with hepatitis C virus infection. Vitamin D impedes T cell proliferation, expression of IFN-γ, expression of IL-2, and CD8 T lymphocyte–mediated cytotoxicity [57].

The NCT04342728 trial is primarily studying whether ascorbic acid and Zn gluconate either alone or in combination can decrease the duration of symptoms in newly diagnosed COVID-19 patients. Ascorbic acid, also known as vitamin C which has potent antioxidant properties, has a very long history (>45 years) in combating or relieving the symptoms of rhinoviruses or common cold [58], and having immune booster properties [59]. Interestingly, various in vitro animal and clinical trials have shown that vitamin C may affect susceptibility to lower respiratory tract infections during the 2003 SARS epidemic [59]. Taking a holistic approach, clinical trial NCT04323228 is using an anti-inflammatory and antioxidant oral nutrition supplement against COVID-19 with rationale that n-3 fatty acids, vitamin A, vitamin C, vitamin E, selenium, and Zn in extraordinary doses may modulate the immune system response and improve the cytokine storm–associated COVID-19 (Readers are referred to specialized articles on antioxidant and/or antiviral properties of various nutrients [4]).

Four clinical studies (NCT04377646, NCT04335084, NCT04384458, NCT04446104 at serial nos. 5, 6, 7, and 8 in Table 1, respectively) are preventive studies, which are studying whether hydroxychloroquine and/or vitamin supplements in combination with Zn are effective as a prophylaxis for asymptomatic healthcare workers/general public. Hydroxychloroquine, derivative of chloroquine having anti-inflammatory, immunosuppressive, and anti-autophagy activities, is an anti-malarial drug and anti-rheumatologic agent. Besides, both hydroxychloroquine and chloroquine constrain pH-dependent steps of SARS-CoV-2 replication by increasing pH in intracellular vesicles [60]. Due to these properties, hydroxychloroquine with Zn and/or azithromycin has been touted as the medicine and as a prophylactic drug for COVID-19 [61–63]. More importantly, chloroquine and hydroxychloroquine work as Zn ionophore, thereby increasing the intracellular Zn concentration [64]. This increase of intracellular Zn concentration will in turn may help in the inhibition of RdRp of SARS-CoV-2 (reviewed in) [51, 61].

The rest of 5 clinical trials (serial nos. 10–15 of Table 1) are aimed at finding better treatment option for COVID-19 by using various combinations of antiviral drugs/dietary supplements with Zn. The results of these studies are still awaited. The common denominator among the above mentioned trials is the use of Zn by virtue of its antioxidant, anti-inflammatory, and antiviral properties [4, 5]. Based on its antioxidant properties, Zn can protect against age-associated macular degeneration, induced by oxidative stress [65]. In general, these properties are the therapeutic basis of the indication of Zn for the treatment of wounds, burns, and acne vulgaris. In addition, Zn is very effective in controlling toxic levels of non-ceruloplasmin copper in Wilson's disease and other copper-related diseases [7]. Acting synergistically, various antioxidants and anti-inflammatory nutrients/drugs combined with antiviral drugs may be beneficial at least in alleviating the COVID-19 symptoms or halting the progression to more severe form of COVID-19. For a clear picture, results of these randomized control trials will be eagerly awaited.

Word of Caution: Zn Toxicity

The recommended dietary allowance (RDA) for Zn is 11 mg/day for men and 8 mg/day for women [66]. Zn absorption is influenced by some foods; e.g., animal proteins result in greater Zn absorption, while phytates reduce its absorption (reviewed in [67]). Zn elimination half-life is in the range of 0.9–1.2 h.

Zn deficiency is far more widespread in population than Zn toxicity (Zn toxicity is very sporadic and occurs very rarely). In comparison to several other metal ions with similar chemical properties, Zn is relatively harmless. Having said that, caution should be practiced against indiscriminate self-medication and chronic use of Zn supplements without proper medical prescription/monitoring as Zn toxicity has also been reported in the literature [68–70]. Acute Zn intoxication is a rare event though exposure to high doses leads to toxic effects. However, it is well established that copper deficiency is associated with taking up large doses of supplemental Zn over extended periods of time [71–73]. In addition, chronic use of Zn as supplements [68] or as a medication [74] can block intestinal absorption of copper [75]. The finely tuned and synchronized systemic homeostasis and efficient regulatory mechanisms keep a check on the cytotoxic doses of exogenous Zn. It is the endogenous Zn that plays a pivotal role in

Table 1 Interventional clinical trials with zinc ± other drugs/dietary supplements for COVID-19

S. no.	Clinical trial study title	ClinicalTrial.Org identifier	Intervention (drug/s)	Zinc dose and duration	Types of intervention/allocation	No. of subjects/age/sex
Clinical trials using zinc with dietary supplements						
1	Impact of zinc and vitamin D_3 supplementation on the survival of aged patients infected with COVID-19	NCT04351490	• Dietary supplement: zinc gluconate • Dietary supplement: 25-OH cholecalciferol	Zinc gluconate 15 mg × 2 per day during 2 months	Treatment/randomized-open label	3140/60 years and older/all sex
2	Coronavirus 2019 (COVID-19)—using ascorbic acid and zinc supplementation	NCT04342728	• Dietary supplement: ascorbic acid • Dietary supplement: zinc gluconate • Dietary supplement: ascorbic acid and zinc gluconate • Other: standard of care	Zinc gluconate 50 mg ± ascorbic acid to be taken daily at bedtime for 10 days	Supportive care/randomized-open label	520/18 years and older/all sex
3	Anti-inflammatory/antioxidant oral nutrition supplementation in COVID-19	NCT04323228	• Dietary supplement: oral nutrition supplement (ONS) enriched in eicosapentaenoic acid, gammalinolenic acid, antioxidants including zinc • Dietary supplement: isocaloric/isonitrogenous ONS	ONS containing 5.7 mg zinc for 14 days	Supportive care/randomized-double masking (participant, care provider)	30/18 years to 65/all sex
4	The study of quadruple therapy zinc, quercetin, bromelain and vitamin C on the clinical outcomes of patients infected with COVID-19	NCT04468139	Drug: quercetin Dietary supplement: bromelain Drug: zinc Drug: vitamin C	Zinc 50 mg orally daily dose	Treatment/single group assignment/masking: none (open label)	60/18 years and older (adult, older adult)/all sex
Preventive clinical trials using zinc with hydroxychloroquine						
5	A study of hydroxychloroquine and zinc in the prevention of COVID-19 infection in military healthcare workers	NCT04377646	• Drug: hydroxychloroquine • Drug: hydroxychloroquine (placebo) • Drug: zinc • Drug: zinc (placebo)	Hydroxychloroquine ± zinc (15 mg) at daily dose up to 2 months	Prevention/randomized/double blind randomized clinical trial with 3 arms-triple masking (participant, care provider, investigator)—random blind allocation	660/18 years to 65 years/all sex
6	A study of hydroxychloroquine, vitamin C, vitamin D, and zinc for the prevention of COVID-19 infection	NCT04335084	• Drug: hydroxychloroquine • Dietary supplement: vitamin C • Dietary supplement: vitamin D • Dietary supplement: zinc	No information available on zinc dose	Prevention/randomized-double masking (participant, investigator)	600/18 years and older/all sex
7	COVID-19 prophylaxis with hydroxychloroquine associated with zinc for high-risk healthcare workers	NCT04384458	• Drug: hydroxychloroquine • Drug: zinc sulfate	Hydroxychloroquine with zinc sulfate (66 mg) daily for 50 days	Prevention/randomized-open label	400/18 years to 70 years/all sex
8	A preventive treatment for migrant workers at high-risk of Covid-19	NCT04446104	• Drug: hydroxychloroquine sulfate • Drug: ivermectin 3 mg tab • Drug: zinc • Drug: povidone-iodine • Dietary supplement: vitamin C	Zinc tablet 80 mg/vitamin C 500 mg daily for 42 days	Prevention/randomized intervention/parallel assignment/open label	5000/21 years to 60 years/all sex
9		NCT04447534	• Drug: chloroquine		Treatment/randomized	

Table 1 (continued)

S. no.	Clinical trial study title	ClinicalTrial.Org identifier	Intervention (drug/s)	Zinc dose and duration	Types of intervention/allocation	No. of subjects/age/ sex
	Zinc with chloroquine/-hydroxychloroquine in treatment of COVID-19		• Drug: zinc	No information available on zinc dose	•Intervention model: parallel assignment •Masking: double (participant, care provider)	200/18 years and older/all sex
Treatment clinical trials using zinc with antiviral drugs/dietary supplements						
10	Hydroxychloroquine and zinc with either azithromycin or doxycycline for treatment of COVID-19 in outpatient setting	NCT04370782	• Drug: hydroxychloroquine • Drug: azithromycin • Drug: zinc sulfate • Drug: doxycycline	Zinc sulfate 220 mg once daily for 5 days	Treatment/randomized-open label	750/30 years and older/all sex
11	A study of quintuple therapy to treat COVID-19 infection	NCT04334512	• Drug: hydroxychloroquine • Drug: azithromycin • Dietary supplement: vitamin C • Dietary supplement: vitamin D • Dietary supplement: zinc	No information available on zinc dose	Treatment/randomized-double masking (participant, investigator)	600/18 years and older/all sex
12	International ALLIANCE study of therapies to prevent progression of COVID-19	NCT04395768	• Dietary supplement: vitamin C • Drug: hydroxychloroquine • Drug: azithromycin • Dietary supplement: zinc citrate • Dietary supplement: vitamin D_3 • Dietary supplement: vitamin B_{12}	Zinc citrate (30 mg) daily	Treatment/randomized-double masking (investigator, outcomes assessor)	200/18 years and older
13	New antiviral drugs for treatment of COVID-19	NCT04392427	• Drug: treatment group: will receive a combination of nitazoxanide, ribavirin, ivermectin, and zinc	No information available on zinc dose	Treatment/randomized-single masking (outcomes assessor)	100/12 years and older/all sex
14	Efficacy of subcutaneous ivermectin with or without zinc and *Nigella sativa* in COVID-19 patients	NCT04472585	• Drug: *Nigella sativa*/black cumin • Drug: ivermectin injectable solution • Other: placebo • Drug: zinc	Zinc sulfate 20 mg 3 times a day	Treatment/randomized, controlled, multi-armed, open-label, interventional study Masking: quadruple (participant, care provider, investigator, outcomes assessor)	40/18 years and older/all sex
15	Trial of combination therapy to treat COVID-19 infection	NCT04482686	• Drug: ivermectin • Drug: doxycycline Hcl • Dietary supplement: zinc • Dietary supplement: vitamin D_3 • Dietary supplement: vitamin C	No information available on zinc dose	Treatment/randomized intervention model/parallel assignment/masking: triple (participant, care provider, investigator)	300/18 years to 75 years/all sex

HCQ, hydroxychloroquine; *ONS*, oral nutrition supplement

Zn-induced cytotoxic events in single cells. Brain, in particular, is very sensitive to Zn toxicity.

Zinc as a Two-Edged Sword

Using Zn as a supplement or treatment adjuvant is a two-edged sword. Large doses of Zn (300 mg/day) over 6 weeks can affect the immune response, impairing lymphocyte, and neutrophil function [76]. On this basis, a concern had arisen that long-term Zn treatment can cause suppression of the immune system. On the similar lines, using excess Zn supplementation as an antiviral therapy can also do harm to the immune system. Maywald et al. have shown in an in vitro study that excess Zn supplementation can in fact reduce the expression of IFN-γ (a key cytokine for T-helper-1 immune reactions) by reducing interferon regulatory factor 1 expression in regulatory T cells [77]. However, it has been shown for elderly persons that zinc supplementation increased interferon-alpha (IFN-α) production by leukocytes in vitro as compared to younger subjects [78]. This observation necessitates the need to continuously monitor the levels of Zn to obtain maximum therapeutic efficacy. Cellular Zn intake can be improved by ionophores including chloroquine and some of its derivatives such as hydroxychloroquine [64]. Alternatively, natural ionophores of potential use with a good tolerability profile are quercetin and epigallocatechin gallate [79].

Summary and Future Directions

With no slowing down of COVID-19 pandemic as indicated by 18 million confirmed cases including 0.69 million deaths worldwide as of August 4, 2020 (https://covid19.who.int/), researchers are seeking ways in which to potentially protect people from the SARS-CoV-2 or to alleviate its effects once caught. One such means is Zn therapy in addition to other antiviral drugs. Zn is well tolerated and it is best known for its antioxidant, anti-inflammatory, immunomodulatory, and antiviral activities, the latter possibly mediated by its ability to inhibit RNA virus RdRp [51], thus protecting the body's cells and tissues from viral infection, oxidative damage, and dysfunction. Based on the current knowledge of beneficial and harmful effects of Zn (Fig. 1), it can be safely concluded that risk to reward ratio is in favor of Zn supplementation in COVID-19. However, the clinical and preclinical data on this aspect is very scanty now and results of current clinical trials employing Zn in COVID-19 can somehow shed more light on the efficacy of Zn against viral infections in vivo. The investigators of the different studies hope to complete the trials in the near future.

Basic and experimental research is still at infancy stage with regard to antiviral mechanisms, clinical benefits, and optimal dose of Zn supplementation as a therapeutic treatment as well as a preventative measure for viral infections including SARS-CoV-2. Generous funding is the need of hour to rigorously pursue this aspect of basic research and provide conclusive evidence to the clinical trials and assumptions based on current knowledge. Although the findings of these trials will be too late for the many thousands of people currently infected with COVID-19, these studies will offer valued data as to the potential alleviation of symptoms by adjunct Zn during future viral/COVID-19 outbreaks and provide platform for future research avenues.

Acknowledgments The authors duly acknowledge the WHO, NCBI, and NIH for providing free online scientific research articles/information/clinical trials pertaining to COVID-19.

Authors' Contributions All authors contributed to the preparation of this review.

Compliance with Ethical Standards

Competing Interests Dr. Rosanna Squitti is the Chief Scientific Officer of IGEA Pharma N.V.; she has some shares in IGEA Pharma N.V. The other authors declare no competing financial interest.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

J Pharm Pharm Sci (www.cspsCanada.org) 23, / 462- 469, 2020

Therapeutic potential of ivermectin as add-on treatment in COVID 19: A systematic review and meta-analysis

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Received, September 18, 2020; Revised, November 17, 2020; Accepted, November21, 2020; Published, November 23, 2020

ABSTRACT -- The current management of COVID-19 is mostly limited to general supportive care and symptomatic treatment. Ivermectin is a broad-spectrum anti-parasitic drug used widely for the treatment of onchocerciasis and lymphatic filariasis. Apart from its anti-parasitic effect it also exhibits antiviral activity against a number of viruses both in vitro and in vivo. Hence, we conducted this systematic review and meta-analysis to assess the currently available data on the therapeutic potential of ivermectin for the treatment of COVID-19 as add on therapy. A total of 629 patients were included in the 4 studies and all were COVID-19 RT-PCR positive. Among them, 397 patients received ivermectin along with usual therapy. The random effect model showed the overall pooled OR to be 0.53 (95%CI: 0.29 to0.96) for the primary outcome (all-cause mortality) which was statistically significant (P=0.04). Similarly, the random effect model revealed that adding ivermectin led to significant clinical improvement compared to usual therapy (OR=1.98, 95% CI: 1.11 to 3.53, P=0.02). However, this should be inferred cautiously as the quality of evidence is very low. Currently, many clinical trials are on-going, and definitive evidence for repurposing this drug for COVID-19 patients will emerge only in the future.

INTRODUCTION:

Since the beginning of the outbreak in December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a cause of global concern (1,2). Remdesivir, favipiravir, chloroquine, hydroxychloroquine, and azithromycin are some of the drugs commonly being used for the treatment of COVID-19 infection but with variable success (3-6). The current management is mostly limited to general supportive care and symptomatic treatment.

Recent reviews have discussed theeffectiveness of multiple classes of drugs including antimalarials, antivirals, antibiotics, corticosteroids and monoclonal antibodies in COVID-19 (7,8). However, the utility of ivermectin, a broad-spectrum anti-parasitic drug used widely for the treatment of onchocerciasis and lymphatic filariasis has largely been overlooked (9,10). Apart from its anti-parasitic effect it also exhibits antiviral activity against a number of viruses both *in vitro* and *in vivo* (11-13). The antiviral activity is purportedly due to the inhibition of importin (IMP) α/β Integrase which helps in the nuclear import and propagation of infection of RNA viruses (14,15). Based on this, researchers have proposed the use of ivermectin as an add on therapy for COVID-19 treatment (16). Few observational studies have been performed to evaluate the effectiveness of ivermectin in the treatment of COVID-19 and have shown favourable result (17-20). However, there is a wide disparity regarding the clinical benefit accrued from this drug. Hence, we conducted this systematic review and meta-analysis to assess the currently available data on the therapeutic potential of ivermectin for the treatment of COVID-19 as add on therapy. This review may provide clinicians an overview of contemporary scientific evidence regarding the therapeutic potential of ivermectin in the clinical management of COVID-19 patients.

MATERIALS AND METHODS:

Development and registration of protocol:

The protocol was written according to PRISMA-P guidelines. The prospective register of systematic

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FOI 26-3132 - Document 5

review (PROSPERO) registration number for the study is CRD42020207299.

Types of studies: All randomized controlled trials (RCTs) and observational studies reporting the use of ivermectin as an add-on therapy in COVID 19 patients in the English language were included. All the included articles reported all-cause mortality as an outcome measure. The study inclusion was not restricted by year of publication, site of study, dose of the drug, and the control arm.

Types of Participants: RT-PCR confirmed COVID-19 adult patients of both genders treated with ivermectin as an add-on therapy were included in the study.

Types of intervention: The intervention in all included studies was the administration of ivermectin in COVID 19 patients along with standard treatment protocol irrespective of the dose, timing, and frequency of administration of ivermectin.

Types of comparator: The comparison was between standard treatment protocol with and without ivermectin.

Outcome measures: Primary outcome were all-cause mortality and any death during the available period of follow up in the studies. Secondary outcomes were 1) time to discharge from the hospital, 2) time to viral clearance by RT-PCR, 3) clinical improvement assessed by the need for respiratory support.

Information source:

PubMed, EMBASE, the Cochrane Library, SCOPUS, and Web of Science were searched for articles on ivermectin as an add-on therapy in COVID 19 from inception till August 31, 2020. The reference lists of retrieved articles were checked for additional studies. For unpublished data, we checked the International Clinical Trials Registry Platform (ICTRP), which is a central database containing trial registration datasets provided by the different international trial registries including ClinicalTrials.gov.Pre-print servers medRxiv and bioRxiv were also searched for pre-print data.

Search strategy: A combination of subject terms and keywords were used and appropriate adjustments of vocabulary and grammar between different databases were done using PICO method. The search used both Medical Subject Headings (MeSH), as well as keyword variants of all relevant terms. A combination of keywords and Boolean operators like "Ivermectin" OR "Anthelminthic" AND "COVID-19" OR "Severe acute respiratory syndrome coronavirus 2" was used for designing the search algorithm.

Data extraction and management: The data were extracted and assessed for quality using the predesigned eligibility criteria following Cochrane Collaboration's guidelines by 3 review authors (RRM, BRM, SD) independently. Any disagreement between them was resolved by the fourth author (BMP). A pre-designed data extraction format was used for the recording of data which included study design, basic information, treatment details, and outcome measures.

Assessment of risk of bias in included studies: Risk of Bias in Non-Randomized Studies - of Interventions (ROBINS-I) for observational studies was used to assess the risk of bias in the included studies.

Data analysis: Cochrane Program Review Manager 5.3 software was used for the Meta-analysis. For dichotomous values, odd's ratio (OR) and 95% confidence interval was expressed in accordance with Cochrane Handbook for Systematic Reviews of Interventions. I^2 statistic was used to check heterogeneity among eligible studies. The random-effect model was used for data synthesis.

Assessment of publication bias: A funnel plot was used to assess the presence of publication bias.

Grade of evidence: GRADE profiler software (V 3.6.1) was used for quality assessment of the evidence.

RESULTS:

Description of studies: A total of 119 studies were found from all database searches. Out of those 91 were from PubMed and 28 from preprint server medRxiv. Search in clinicaltrials.gov resulted in 37 registered studies, among those 2 were completed studies with results. A total of 5 studies were selected for full-text review after screening and removal of duplicates. Other studies were excluded as those were letters to the editor, commentaries, and review articles. Finally, 4 observational studies were included for systematic review and meta-analysis, 3 with the control arm and 1 without control arm. One

of the studies evaluating the prophylactic effect of ivermectin for COVID-19 was excluded from the review. We did not find any published RCT evaluating the effect of ivermectin on COVID-19. Three of the included studies are not peer-reviewed as they were published in preprint server medRxiv.

A total of 629 patients were included in the 4 studies and all were RT-PCR positive. Among them, 397 patients received ivermectin along with usual therapy. Ivermectin treated group had 233 mild cases and 104 moderate to severe cases. Similarly, the usual treatment group had 121 mild and 57 moderate to severe cases. The study by Choudhury *et al* with 60 patients in the ivermectin group and 56 patients in the usual therapy group had not mentioned the severity of the disease. Most of the patients had one or more comorbidities like diabetes, hypertension, and bronchial asthma. The dose of ivermectin varied from 150 to 200 µg/kg body weight administered as a single dose. In the study by Rajtor *et al*, 13 patients also received the 2nd dose of ivermectin. The characteristics of studies included for systematic review and meta-analysis are depicted in Table 1.

Risk of bias in the included studies:

The risk of bias assessment of all studies included in the meta-analysis was carried out for the primary outcome (all-cause mortality) using the risk of bias in non-randomized studies - of interventions (ROBINS-I). The result of the risk of bias assessment of observational studies is depicted in table 2.

Effects of intervention:

The study by Bhattacharya *et al* did not have a control arm for comparison. Therefore, 3 studies were included for measuring the pooled effect of add on ivermectin to usual therapy on the primary and secondary outcomes. The forest plots for the pooled effect are depicted in Figure 1.

Primary outcome (all-cause mortality)

All-cause mortality was reportedly reduced in 2 of the 3 included studies. Test for heterogeneity for the pooled studies was not significant (Chi^2 =0.09, df=1, (P=0.77), I^2=0%) and the random effect model showed the overall pooled OR to be 0.53 (95%CI: 0.29 to 0.96). Sensitivity analysis was not carried out as the test of heterogeneity across the included studies was not significant. The overall result suggests that there was a statistically significant reduction in all-cause mortality with the additional use of ivermectin compared to usual therapy only (P=0.04).

Since the search period mentioned in our protocol registered on PROSPERO was from inception till August 31, 2020, data from the study by Hashim *et al* has not included in the primary meta-analysis as this trial was published in the preprint server in October 2020 (21). In this study, out of 140 COVID-19 patients, 70 were randomized to receive ivermectin plus standard treatment and the rest 70 received standard treatment only. In the ivermectin plus standard treatment arm, the mortality was 2 compared to 6 in the standard treatment only arm. As a secondary analysis, when this study was included, the test for heterogeneity for the pooled studies was not significant (Chi^2 =0.45, df=2, (P=0.80), I^2=0%) and pooled OR was 0.50 (95%CI: 0.29 to 0.88). This suggested that addition of ivermectin significantly reduced the mortality (P= 0.02).

Secondary outcomes:

Clinical improvement assessed by the need for respiratory support:

All the 3 studies included in the meta-analysis reported clinical improvement as assessed by the need for respiratory support until the available follow-up period. The test of heterogeneity was not significant (Chi^2=0.56, df=2, (P=0.76), I^2 =0%). The random effect model revealed that adding ivermectin led to significant clinical improvement compared to usual therapy (OR=1.95, 95% CI: 1.09 to 3.49, P=0.02).

Time to discharge from the hospital:

In the study by Gorial *et al*, the meantime of hospital stay was significantly lower in the ivermectin group than the non-ivermectin group (7.62± 2.75 versus 13.22± 5.90 days, p= 0.00005). But in other studies, there was no significant difference in time to discharge from the hospital between the 2 groups. As the exact data for time to discharge from the hospital within a specified time period was not available in all the studies, the data were not pooled for meta-analysis.

Time to viral clearance by RT-PCR:

The median time to viral clearance was lower in the ivermectin group (7 days) in the study by Gorial *et al*, which was statistically significant (p=0.001)

compared to the non-ivermectin group (12 days). But the study by Choudhury *et al* did not find any significant difference between the 2 groups. The other 2 studies by Rajter *et al* and Bhattacharya *et al* did not report about time to viral clearance.

Publication bias:

Although we created a Funnel plot for publication bias, it could not be assessed as there were only 2 studies where mortality was reported. However, due to the small sample size and large effect we expect that publication bias would be present.

Grade of evidence:

The evidence was assessed using GRADE profiler for all-cause mortality and clinical improvement. There were 54 lower deaths per 1000 population on the addition of ivermectin to usual therapy. Similarly, clinical improvement was achieved in 61 more cases per 1000 population when ivermectin was used as an add-on therapy. However, the grade of evidence was very low for both the outcomes. The detailed analysis of the summary of evidence is depicted in table 3.

DISCUSSION

In this systematic review and meta-analysis, we have synthesized the available evidence on the use of add on ivermectin in COVID-19 patients. We included 3 observational studies for estimating the pooled effect. It is important to note that at the time of analysis, 2 of the included studies were not peer-reviewed and were retrieved from the preprint server. However, one of these, the ICON study by Rajter *et al* has been recently published (18). To the best of our knowledge, this is the first systematic review and meta-analysis on the effectiveness of add-on ivermectin in COVID-19 patients. The pooled estimate suggested a statistically significant reduction in all-cause mortality when ivermectin was added to usual therapy with OR of 0.53 (P= 0.04). Even on secondary analysis after inclusion of the study by Hashim *et al*, the pooled OR did not change meaningfully and was 0.50 (P=0.02). Ivermectin has been hypothesized to exert its effect by inhibiting importin (IMP) α/β Integrase and thereby preventing the propagation of viruses. However, the exact mechanism is still not established.

The OR of 1.98 indicates significant clinical improvement as assessed by the need for respiratory support by using ivermectin as add-on therapy in the management of COVID-19 patients (p= 0.02). However, this should be inferred cautiously as the sample sizes of included studies were relatively small and showing large effect.

Most of the included studies involved mild to moderate cases that might have not required respiratory support. All the included studies were observational in nature and suffered from various biases inherent to such study design. The study by Gorial *et al* used a synthetic matched control group for comparison. Similarly, one of the included studies was a retrospective case-control study. All these factors, therefore, contribute to the overall very low quality of evidence. Additionally, the complication rate and mortality amongst patients with severe disease have been reported to be very high (22). In such patients the effectiveness of add on ivermectin has not yet been explored.

Although add-on ivermectin was associated with a significant reduction in all-cause mortality and clinical improvement it did not translate into early discharge from the hospital or viral clearance. Only the study by Gorial et *al* reported a significant decrease in a hospital stay and viral clearance. This might have been due to differences in the management protocol followed in different countries.

In a recent study whose data has been made publicly available on www.clinical trials.gov , [NCT04422561] prophylactic use of ivermectin has reduced the development of COVID-19 symptoms during 14 days follow up period (7.4% in ivermectin group compared to 58.4% in the control group). This drug has traditionally been used in the mass prophylaxis programs of filariasis, onchocerciasis and is generally safe and well-tolerated. In all the studies included in this review, the safety profile of ivermectin was reported to be favourable.

STRENGTH AND LIMITATIONS:

This is the first meta-analysis on the effectiveness of ivermectin as an add-on therapy in COVID-19 using a comprehensive search strategy and assessing the strength of evidence using GRADE profiler. Limitations include small observational studies with confounders and possible publication bias thus providing very low-quality evidence.

FOI 26-3132 - Document 5

Table 1: Characteristics of included studies

Study, Year, Country	Patient characteristics		Primary outcome, All-cause mortality		Secondary outcome						Additional comment
					Time to discharge		Clinical improvement		Viral clearance		
	IVM	No IVM	IVM	No IVM	IVM	No IVM	IVM	No IVM	IVM	No IVM	
Gorial *et al*, 2020, Iraq (17), [From preprint server]	n= 16; age, 44.9±10.6; male/female, 11/5; 9 mild,7 moderate	n= 71; age- 45.2± 18.5; Male/Female, 52/19; 40 Mild, 31Moderate	0	2	Mean, 7.62± 2.75	Mean- 13.22± 5.90	16	69	Median- 7 (95% CI 6-11)	Median- 12 (95% CI 10-15)	Matched control study; exclusion- severe covid-19; follow up, 23 days, IVM dose- 200µg/ kg, single dose; received hydroxychloroquine, azithromycin
Rajter *et al*, 2020, USA (18)	n= 173; age- 60.2± 17.6; male/female, 89/84; mild,124; moderate to severe, 49	n= 107; age- 58.6± 18.5; Male/Female, 64/43, mild, 81; moderate to severe, 26	26	27	Median (IQR), 7 (4-13)	Median (IQR), 7 (4-10)	147	80	Not reported	Not reported	Retrospective study; follow up, 7 to 64 days; 200 µg/ kg IVM, single dose; 13 received 2nd dose of IVM. also received hydroxychloroquine azithromycin
Chowdhury *et al*, 2020, Bangladesh (19)	n= 60; male/female, 43/17; asymptomatic, 11; symptomatic,49	n=56; male/female, 47/9; asymptomatic,14; symptomatic,42	0	0	Mean- 8.9 days	Mean- 9.33 days	60	54	Mean- 5.93 days	Mean- 7 days	Comparative observational study; IVM dose- 200 µg/ kg, single dose; IVM group also received doxycycline; non IVM treatment group received hydroxychloroquine, azithromycin
Bhattacharya *et al*, 2020, India (20)	n=148; mean age- 57.57male/female, 72/76; mild,100; moderate to severe, 48	No comparator	2		Average- 12 days		146				Retrospective case series; IVM dose- 200 µg/ kg, single dose. Also received n-acetylcysteine and atorvastatin

IVM- Ivermectin, IQR- Inter quartile range, µg/kg- Microgram/ kilogram body weight.

FOI 26-3132 - Document 5

Table 2: Risk of bias assessment in included studies (ROBINS I)

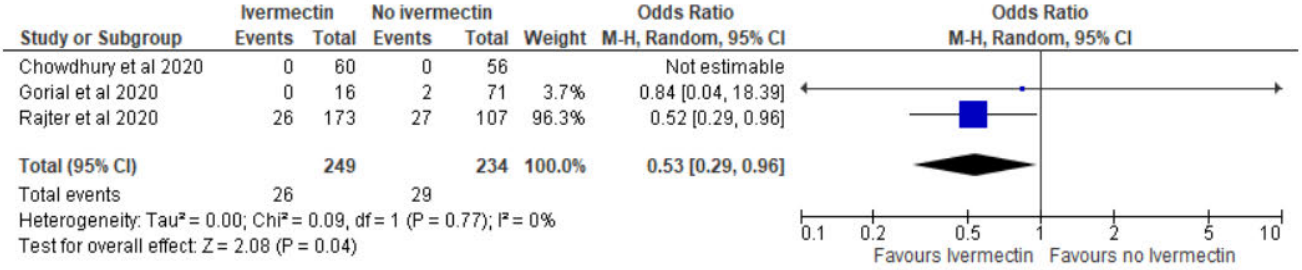
Study	D1	D2	D3	D4	D5	D6	D7	Overall
Chowdhury *et al,* 2020								
Gorial *et al*, 2020								
Rajter *et al*, 2020								

Domains:
D1: Bias due to confounding
D2: Bias in selection of participants into the study
D3: Bias in classification of intervention
D4: Bias due to deviation from intended intervention
D5: Bias due to missing data
D6: Bias in measurement of outcomes
D7: Bias in selection of reported outcome

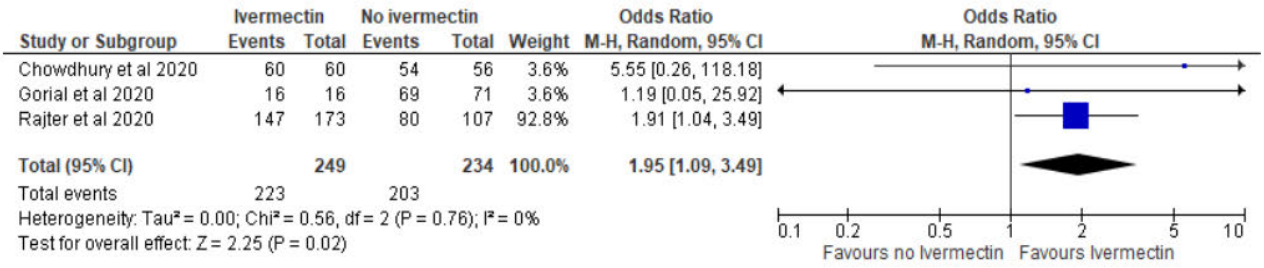
Low:
Moderate:
Serious:
Critical:
No inference:

Figure 1: Forest plots of effectiveness of add-on ivermectin therapy

a. All-cause mortality



b. Clinical improvement



FOI 26-3132 - Document 5

Table III: Grade of evidence for included studies

Ivermectin for COVID-19, Bibliography: Meta-analysis

Outcomes	No of Participants (studies) Follow up	Quality of the evidence(GRADE)	Relative effect (95% CI)	Anticipated absolute effects	
				Risk with control	Risk difference with Ivermectin (95% CI)
All-cause mortality	483 (3 studies)	⨁⊖⊖⊖ VERY LOW[1,2,3,4,5] due to risk of bias, inconsistency, indirectness, publication bias, large effect	OR 0.53 (0.29 to 0.97)	Study population 125 per 1000 moderate effect	54 fewer per 1000 (from 3 fewer to 85 fewer)
Secondary outcomes	No of Participants (studies) Follow up	Quality of the evidence (GRADE)	Relative effect (95% CI)	Anticipated absolute effects,	
				Risk with control	Risk difference with Ivermectin (95% CI)
Clinical improvement	483 (3 studies)	⨁⊖⊖⊖ VERY LOW[1,2,3,4,5] due to risk of bias, inconsistency, indirectness, publication bias, large effect	OR 1.98 (1.11 to 3.53)	Study population 868 per 1000 moderate effect	61 more per 1000 (from 12 more to 91 more)

*The basis for the assumed risk (e.g. the median control group risk across studies) is provided in footnotes. The corresponding risk its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and it CI). CI: Confidence interval; OR: Odds ratio;

GRADE Working Group grades of evidence; High quality: Further research is very unlikely to change our confidence in the estim effect; Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and ma change the estimate; Low quality: Further research is very likely to have an important impact on our confidence in the estimate of and is likely to change the estimate; Very low quality: We are very uncertain about the estimate.

[1] Bias due to confounding: [2] Due to study types,[3] Due to retrospective study, [4] Due to small number of studies' [5] due to less numb study and small sample size

CONCLUSION

Ivermectin is an established drug with a long history of clinical use and with minimal safety concern. Recent observational studies have reported the effectiveness of this drug as add-on therapy in patients with COVID-19. Our meta-analysis also supports this finding and suggests the modest utility of ivermectin in reducing all-cause mortality and improving clinical outcomes. Currently, many clinical trials are on-going, and definitive evidence for repurposing this drug for COVID-19 patients will emerge only in the future.

AUTHOR CONTRIBUTION: The concept was developed by RRM and BMP. BRM, SD, and RRM carried out the search, data extraction, and quality assessment. Any disagreement was resolved by BMP. Statistical analysis and inference was done by BMP and BRM. The manuscript was written by RRM, BRM, BMP, and SD. All authors approved the final version for publication.

CONFLICTS OF INTEREST: There are no conflicts of interest.

FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Title: Zinc^{2+} ion inhibits SARS-CoV-2 main protease and viral replication *in vitro*.

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Abstract:

Zinc deficiency is linked to poor prognosis in COVID-19 patients while clinical trials with Zinc demonstrate better clinical outcome. The molecular target and mechanistic details of anti-coronaviral (SARS-CoV2) activity of Zinc remain obscure. We show that ionic Zinc not only inhibits SARS-CoV-2 main protease (Mpro) with nanomolar affinity, but also viral replication. We present the first crystal structure of Mpro-$Zinc^{2+}$ complex at 1.9 Å and provide the structural basis of viral replication inhibition.

COVID-19 pandemic caused by SARS-CoV-2 is a major clinical challenge[1,2,3]. Lower serum Zinc concentration at the time of admission of COVID-19 patients correlates with severe clinical presentations; an extended duration to recovery, higher morbidity, and a higher mortality in elderly[4,5]. However, clinical trials with Zinc and ionophore show positive clinical outcome with a decreased rate of mortality, and transfer to hospice[6,7,8].

Zinc plays several key roles in biological systems viz. structural, catalytic, regulatory and signalling events[9,10,11]. Further, Zinc exhibits anti-viral properties[12], including anti-SARS-CoV. SARS-CoV Main protease (Mpro)[13] and RNA dependent RNA polymerase (RDRP)[14] are potential key molecular targets of Zinc. However, the structure of SARS-CoV-2 RDRP[15] suggests a structural role for Zinc rather than an inhibitory one. Notably, detailed kinetics and mechanism of ionic Zinc targeting SARS-CoV-2 Mpro is lacking.

We first studied one on one binding kinetics of Zinc acetate with purified SARS-CoV-2 Mpro using Surface Plasmon Resonance (SPR). Zinc binds to SARS-CoV-2 Mpro with an association rate constant (ka) of 8,930±30 $M^{-1}s^{-1}$ and the dissociation rate constant (kd) of 0.01755±10 s^{-1}, and an equilibrium dissociation constant (KD) of 1.965E-06 M. (**Fig. 1a**) The half-life (t1/2=ln [0.5]/kd) of Zinc-Mpro complex is computed to be ~10s. We then assessed the inhibitory effects of $Zinc^{2+}$ binding on the proteolytic activity of SARS-COV-2 Mpro in the presence of Zinc acetate. We obtained an IC_{50} value of 325.1 ± 5.1 nM with complete inhibition at 6.25 µM and

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above (**Fig. 1b**). We also tested Zinc glycinate and Zinc gluconate complexes, which are available as Zinc supplements in the market and are also investigated in COVID-19 clinical trials[16], and obtained IC_{50} values of 279.35±17.95 nM and 405.25±0.45 nM, respectively (**Supplementary Figure 1, a and b**). Reversibility of $Zinc^{2+}$-mediated inhibition was tested by first inhibiting the enzyme with 500 nM Zinc acetate, and then initiating the reaction with a substrate peptide, followed by addition of EDTA to regain the enzyme activity by chelating $Zinc^{2+}$ ions. We find that Zinc inhibition is completely reversible by EDTA (**Supplementary Figure 1, c**), suggesting that inhibition by the metal ion is not because of oxidation of catalytic cysteine (Cys145).

To further understand the structural basis of SARS-CoV-2 Mpro inhibition by $Zinc^{2+}$ ion, we solved the crystal structure of $Zinc^{2+}$ bound complex at 1.9 Å (**Supplementary Table 2**). The asymmetric unit contains a dimer of Mpro in space group $P2_12_12_1$ (**Fig. 1c**). An unambiguous electron density for $Zinc^{2+}$ (**Fig. 1d, e**) shows that the metal ion is coordinated by the catalytic dyad His41 and Cys145, which is absent in the control datasets collected for apo-enzyme crystals grown in the same condition. $Zinc^{2+}$-bound complex shows a tetrahedral coordination geometry at the Mpro active site by coordinating with two water molecules that are absent in the apo-enzyme structure (**Fig. 1c**). Distortion in the tetrahedral geometry observed is attributed to the presence of heterogeneous atoms; sulphur (Cys145-SG) and nitrogen (His41-NE2) in the inhibited complex. A 180º flip of the imidazole ring of His41 brings NE2 closer to $Zinc^{2+}$ with an inter-atomic distance of 1.94 Å to form a coordinate bond. The interatomic distance between catalytic Cys145 and Zinc is 2.36 Å consistent with observed bound complexes. Two structural water molecules W1 and W2 (PDB: 7DK1; HETATM 5028 and 5031, respectively) coordinate $Zinc^{2+}$ at an inter-atomic distance of 2.23 Å and 1.98 Å, respectively, to satisfy the tetrahedral geometry (**Fig. 1c**). The coordination of $Zinc^{2+}$ with the catalytic dyad is expected to prevent a nucleophilic attack on the carbonyl moiety of the amide bond in polyprotein substrate. We hypothesize that the two strongly coordinated water molecules impart stability to the $Zinc^{2+}$ inhibited complex.

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To gain deeper insights into the stability of Mpro- $Zinc^{2+}$ complex, we simulated the complex for 1 µs at 300K, keeping the coordinating waters, W1 and W2. During the simulation, $Zinc^{2+}$ remains strongly bound to the active site via metal coordination bonds with His41 (NE2) and Cys145 (SG) with an interatomic distance of 1.951±0.031 and 2.518±0.031 Å, respectively, throughout the 1µs time frame. The mobility of $Zinc^{2+}$ ion is restricted with a mean RMSD of 0.920 Å (**Fig. 1f**) in accordance with the dynamics of side chains of coordinating catalytic dyad. Visualization of MD simulation trajectory shows (**Supplementary Movie**) that coordinating water molecules W1 and W2 remain bound to $Zinc^{2+}$ throughout the simulation, and help maintain the tetrahedral geometry observed in complex crystal structure (**Fig. 1g**).

We further assessed the inhibitory potential of Zinc acetate, Zinc glycinate and Zinc gluconate against SARS-COV-2. Infected Vero E6 cells were treated with all the three Zinc salts at their respective maximum non-toxic concentrations (MNTD) for 48 hours. The MNTDs used for the three compounds were 100 µM for Zinc acetate and Zinc gluconate and 70 µM for Zinc glycinate. We observed that Zinc acetate treatment resulted in more than 50% reduction of viral titre, as compared to the untreated infected cells (**Fig. 2a**). Based on these results, we determined the IC_{50} of Zinc acetate to be 3.227 µM (**Fig 2b**). Surprisingly, Zinc glycinate and Zinc gluconate failed to inhibit viral replication in standard antiviral assays at non-toxic concentrations albeit showing effective enzyme inhibition *in vitro*. Quercetin, a natural Zinc ionophore, increases the bioavailability of Zinc in treated cells[17], which prompted us to ask whether an increased bioavailability of $Zinc^{2+}$ results in enhanced inhibition of viral replication. To test this, we mixed Zinc acetate with Quercetin at 1:2 molar ratio[18] at non-toxic concentrations (**Supplementary Figure 2**) and tested the antiviral activity against SARS-CoV-2. We observed >2-fold viral inhibition in the presence of Quercetin (**Fig. 2c**).

Taken together, our data strongly suggest an inhibitory role for ionic Zinc[10], wherein it inhibits SARS-CoV-2 Mpro enzyme activity, supported by complex crystal structure and subsequent inhibition of viral replication *in vitro.* Known crystal structures of Zinc conjugates such as N-ethyl-n-phenyl-dithiocarbamic acid (EPDTC), JMF1600, and Zinc-pyrithione in complex with 3C-like (Mpro) proteases from coronaviruses[19], including SARS-CoV[20] and SARS-CoV-2[21],

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show a similar mode of metal ion coordination with the catalytic dyad (**Supplementary Figure 3**). However, the $Zinc^{2+}$ inhibited SARS-CoV-2 Mpro enzyme structure presented in this study clearly suggests that ionic form of Zinc alone is capable of inhibiting the enzyme, by forming a stable complex at the active site with the help of two water molecules, previously unknown. We further show Zinc complexes; Zinc glycinate and Zinc gluconate failed to produce any antiviral effects in our cell culture experiments, in agreement with a recent clinical trial data[16] that show no significant effect of Zinc gluconate in clinical outcome in COVID-19 patients. Most notably, we show that Zinc ionophore Quercetin aids in inhibition of SARS-CoV-2 replication as it increases the intracellular concentration of Zinc[17]. Our data support the findings that a combination of Zinc salt, which provides ionic Zinc, with ionophores[6-8], may have a better clinical outcome in COVID-19 therapy. As the $Zinc^{2+}$ coordinating catalytic dyad; Cysteine and Histidine, is present across all coronaviral 3C-like protease (Mpro) active sites, including the ones coded by virulent strains of SARS-CoV-2, the mode of $Zinc^{2+}$ inhibition is expected to be conserved. Short half-life (~10s) of Mpro-$Zinc^{2+}$ complex, fast association and dissociation rates, and water-soluble nature of Zinc acetate suggest that constant doses of Zinc-ionophore combination may be required for effective inhibition of SARS-CoV-2 Mpro.

Data accessibility: Mpro-$Zinc^{2+}$ complex coordinates are available at PDB: 7DK1. X-ray raw data is available from Integrated Resource for Reproducibility in Macromolecular Crystallography (IRRMC) repository (https://proteindiffraction.org/).

Acknowledgement: We thank Prof. Rolf Hilgenfeld, Institute of Biochemistry, University of Lübeck, Lübeck, Germany for providing the expression construct for SARS-CoV-2 Mpro. We thank the beamline staff at the Elettra XRD2 particularly Raghurama P. Hegde and Annie Heroux for beamline support. Access to the XRD2 beamline at the Elettra synchrotron, Trieste was made possible through grant-in-aid from the Department of Science and Technology, India, vide grant number DSTO-1668. The following reagent was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52281. We thank Prof. Ramesh Sonti, former

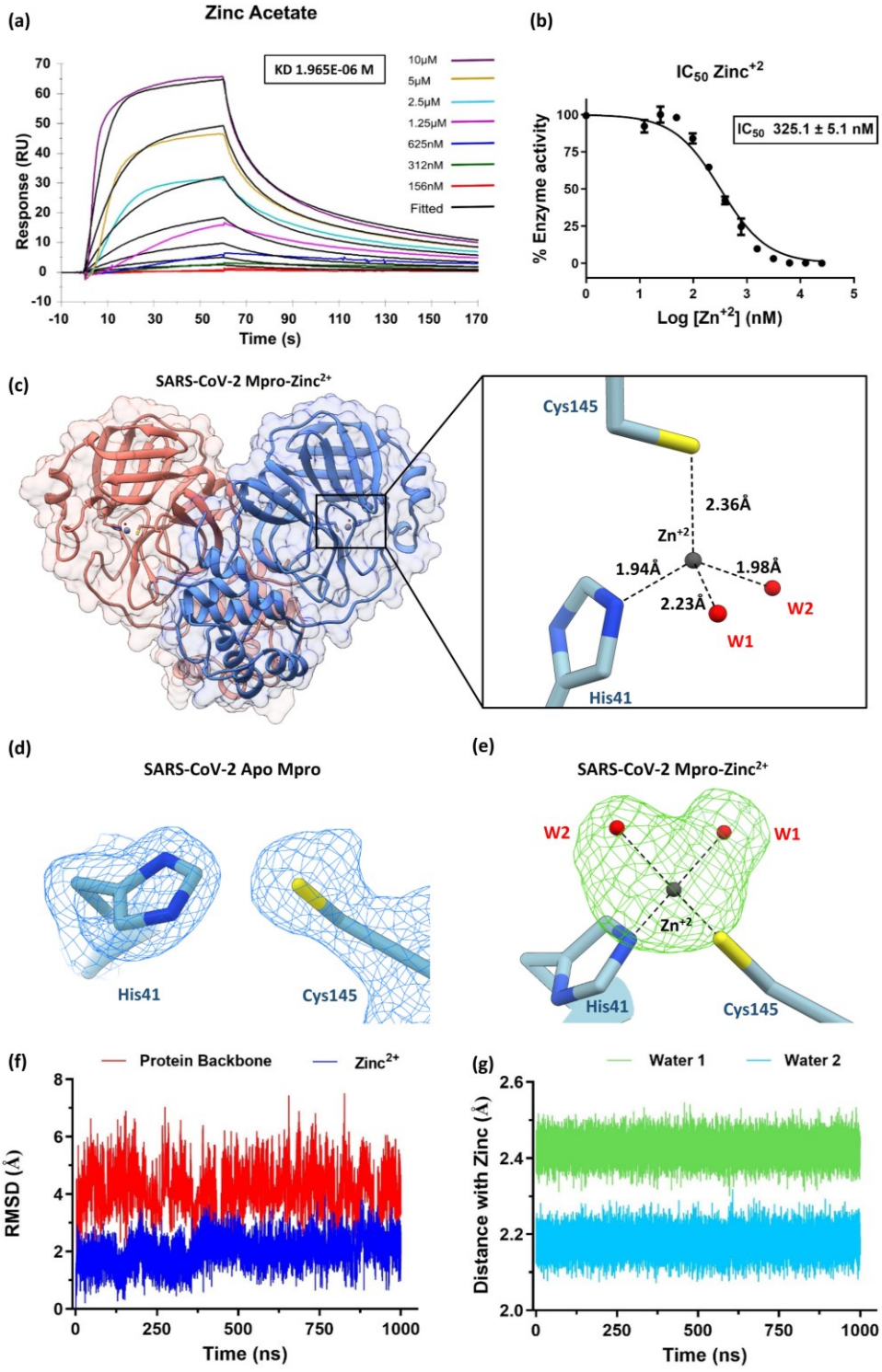
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Director, NIPGR, New Delhi for access to Biacore T-200. This work was supported by ICGEB core grant and Govt. of India DST-SERB IRHPA grant: IPA/2020/000285 to AA and S. Sunil.

Author contribution: LP, WK, SK, KS purified Mpro and performed biochemical and SPR experiments and SD and JKT helped with SPR experiments and data analysis. LP and KS crystallized, collected X-ray data and solved the structure. LP and SS performed MD simulation and analysis. AG performed cytotoxicity assays. AK, DV, AH and S. Sunil performed anti-viral assays and analysed the data. RH provided inputs to biochemical assays. AA coordinated the work and LP and AA wrote the manuscript with inputs from all the authors.

Conflict of interest: The authors declare no conflict of interest.

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152

153 **Figure 1. Zinc^{2+} binds at the active site and inhibits SARS-CoV-2 Mpro enzyme activity:**

154 **(a)** Interaction kinetics of Zinc^{2+} with immobilized Mpro using surface plasmon resonance (SPR)

155 shows affinity (KD) of 1.96 μM. Coloured lines indicate various concentrations of Zinc acetate.

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(b) IC_{50} and concentration dependent inhibition of Mpro by $Zinc^{2+}$ ion. **(c)** Complex crystals structure of Mpro dimer with Zinc (grey sphere) bound at the active site of both protomers. On the right, catalytic dyad Cys145 and His41 of Mpro is shown with bound Zinc in tetrahedral coordination geometry. **(d)** Electron density map (2Fo-Fc) contoured at 1 σ showing the catalytic dyad in Apo-Mpro **(e)** Omit difference map (Fo-Fc contoured at 3 σ) shows unambiguous density (green) for $Zinc^{2+}$ (grey) and two metal-ion coordinating structural water molecules (red)**. (f)** 1µs MD simulation Mpro-$Zinc^{2+}$ complex shows low RMSD of $Zinc^{2+}$ (blue) and protein backbone (red) indicating stability of inhibited state **(Supplementary video) (g)** Distance plot shows less fluctuations in inter-atomic distances between $Zinc^{2+}$ and two coordinating water molecules during the course of simulation, representing stable metal ion-water interactions in the inhibitory role of $Zinc^{2+}$.

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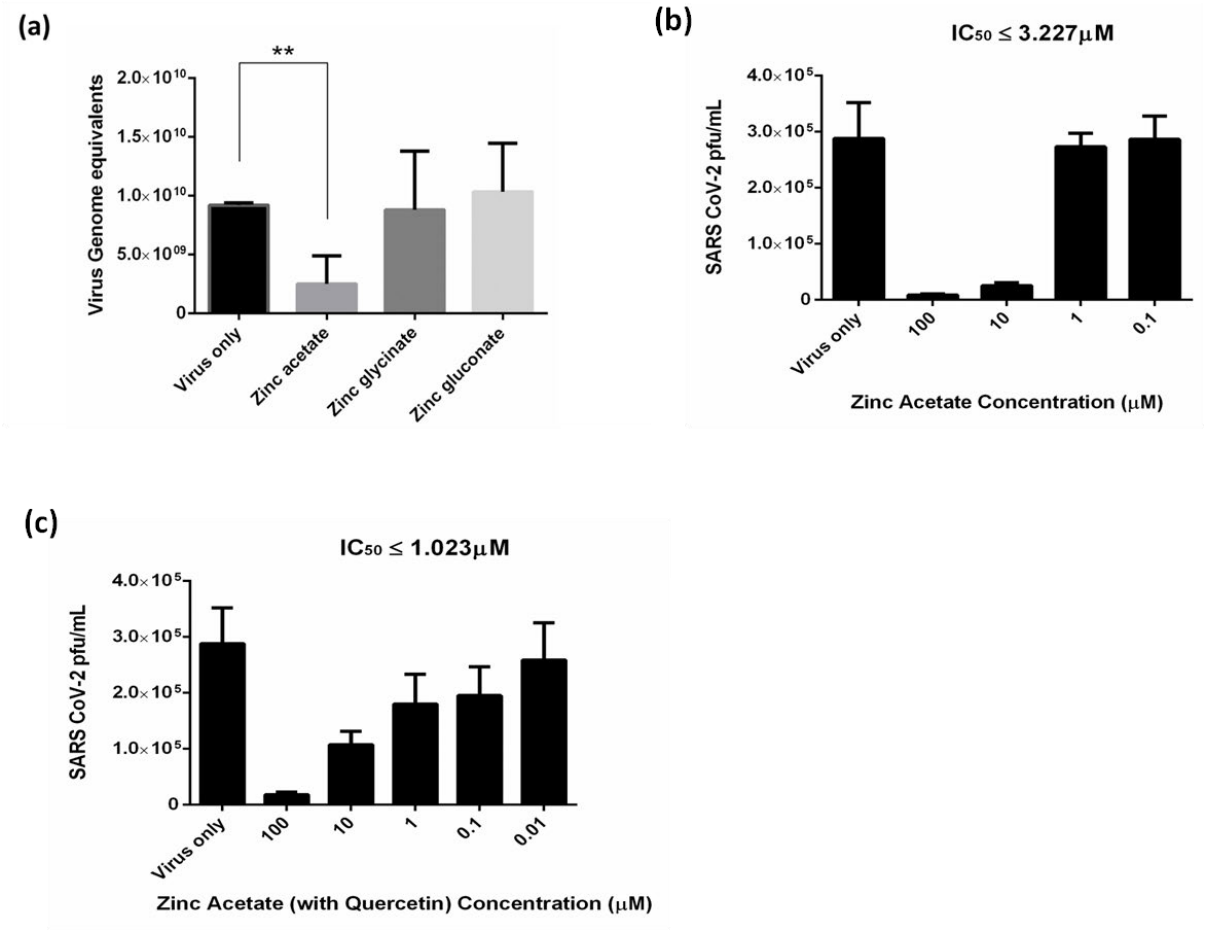


Figure 2. Anti-SARS-CoV-2 activity of Zinc and its complexes with ionophore in infected Vero E6 cells. a) Zinc acetate inhibits SARS-CoV-2 replication in Vero E6 cells as determined using qRT-PCR. Treatment with Zinc acetate (100 μM) for 48 h resulted in >50% reduction of viral titre while the Zinc glycinate and Zinc gluconate complexes did not show significant reduction. **(b)** IC_{50} determination using varying concentrations of Zinc for 48 h followed by viral quantification using plaque assay. **(c)** Viral inhibition by Zinc acetate and Quercetin mixture (1:2 M ratio). Mean percentage reduction of SARS-CoV-2 is indicated within the bars. The antiviral experiments were repeated at least twice, and each experiment included at least three replicates. Statistical significance was determined using Student's t-test (n ≥ 2 biological replicates).

FOI 26-3132 - Document 5

Zinc in Human Health: Effect of Zinc on Immune Cells

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Although the essentiality of zinc for plants and animals has been known for many decades, the essentiality of zinc for humans was recognized only 40 years ago in the Middle East. The zinc-deficient patients had severe immune dysfunctions, inasmuch as they died of intercurrent infections by the time they were 25 years of age. In our studies in an experimental human model of zinc deficiency, we documented decreased serum testosterone level, oligospermia, severe immune dysfunctions mainly affecting T helper cells, hyperammonemia, neurosensory disorders, and decreased lean body mass. It appears that zinc deficiency is prevalent in the developing world and as many as two billion subjects may be growth retarded due to zinc deficiency. Besides growth retardation and immune dysfunctions, cognitive impairment due to zinc deficiency also has been reported recently. Our studies in the cell culture models showed that the activation of many zinc-dependent enzymes and transcription factors were adversely affected due to zinc deficiency. In HUT-78 (T helper 0 (Th_0) cell line), we showed that a decrease in gene expression of interleukin-2 (IL-2) and IL-2 receptor α (IL-2Rα) were due to decreased activation of nuclear factor-κB (NF-κB) in zinc deficient cells. Decreased NF-κB activation in HUT-78 due to zinc deficiency was due to decreased binding of NF-κB to DNA, decreased level of NF-κB p105 (the precursor of NF-κB p50) mRNA, decreased κB inhibitory protein (IκB) phosphorylation, and decreased Iκκ. These effects of zinc were cell specific. Zinc also is an antioxidant and has anti-inflammatory actions. The therapeutic roles of zinc in acute infantile diarrhea, acrodermatitis enteropathica, prevention of blindness in patients with age-related macular degeneration, and treatment of common cold with zinc have been reported. In HL-60 cells (promyelocytic leukemia cell line), zinc enhances the upregulation of A20 mRNA, which, via TRAF pathway, decreases NF-κB activation, leading to decreased gene expression and generation of tumor necrosis factor-α (TNF-α), IL-1β, and IL-8. We have reported recently that in both young adults and elderly subjects, zinc supplementation decreased oxidative stress markers and generation of inflammatory cytokines.

Online address: http://www.molmed.org
doi: 10.2119/2008-00033.Prasad

INTRODUCTION

In 1869, Raulin (1) first showed that zinc was required for the growth of Aspergillus niger. In 1934, Todd *et al*. (2) reported that zinc was essential for the growth of rats. Although the essentiality of zinc for animals was established, its ubiquity made it seem improbable that zinc deficiency in humans could lead to any significant clinical problem. During the past 40 years, however, it has become apparent that deficiency of zinc in humans is quite prevalent and may affect over two billion subjects in the developing world (3–5). Here, we review some of the clinical manifestations of zinc deficiency in humans, with particular emphasis on our observations in experimental human models of this syndrome.

CLINICAL MANIFESTATIONS OF ZINC DEFICIENCY

During the past four decades, a spectrum of clinical deficiency of zinc in human subjects has emerged (6). On the one hand, the manifestations of zinc deficiency may be severe; and, on the other end of the spectrum, zinc deficiency may be mild or marginal. A severe deficiency of zinc has been reported to occur in patients with acrodermatitis enteropathica (a genetic disorder), following total parenteral nutrition (TPN) without zinc, following excessive use of alcohol, and following penicillamine therapy. The manifestations of severe zinc deficiency in humans include bullous pustular dermatitis, alopecia, diarrhea, emotional disorder, weight loss, intercurrent infections due to cell-mediated immune dysfunctions, hypogonadism in males, neurosensory disorders, and problems with healing of ulcers. If this condition is unrecognized and untreated, it becomes fatal.

The manifestations of a moderate deficiency of zinc include growth retardation and male hypogonadism in adolescents, rough skin, poor appetite, mental lethargy, delayed wound healing, cell-mediated immune dysfunctions, and abnormal neurosensory changes.

In our studies in the experimental human model in whom only a mild deficiency of zinc in males was induced by dietary means, decreased serum testosterone level, oligospermia, decreased natural killer (NK) cell activity, decreased interleukin-2 (IL-2) production, decreased thymulin activity, hyperammonemia, hypogeusia, de-

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Submitted March 17, 2008; Accepted for publication March 24, 2008; Epub (www.molmed.org) ahead of print April 3, 2008.

Received: 6 December 2020 | Revised: 18 January 2021 | Accepted: 20 January 2021

FOI 26-3132 - Document 5

DOI: 10.1002/jmv.26895

SHORT COMMUNICATION

JOURNAL OF MEDICAL VIROLOGY WILEY

A pilot double-blind safety and feasibility randomized controlled trial of high-dose intravenous zinc in hospitalized COVID-19 patients

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Abstract

Zinc inhibits replication of the SARS-CoV virus. We aimed to evaluate the safety, feasibility, and biological effect of administering high-dose intravenous zinc (HDIVZn) to patients with COVID-19. We performed a Phase IIa double-blind, randomized controlled trial to compare HDIVZn to placebo in hospitalized patients with COVID-19. We administered trial treatment per day for a maximum of 7 days until either death or hospital discharge. We measured zinc concentration at baseline and during treatment and observed patients for any significant side effects. For eligible patients, we randomized and administered treatment to 33 adult participants to either HDIVZn (n = 15) or placebo (n = 18). We observed no serious adverse events throughout the study for a total of 94 HDIVZn administrations. However, three participants in the HDIVZn group reported infusion site irritation. Mean serum zinc on Day 1 in the placebo, and the HDIVZn group was 6.9 ± 1.1 and 7.7 ± 1.6 μmol/l, respectively, consistent with zinc deficiency. HDIVZn, but not placebo, increased serum zinc levels above the deficiency cutoff of 10.7 μmol/l (p < .001) on Day 6. Our study did not reach its target enrollment because stringent public health measures markedly reduced patient hospitalizations. Hospitalized COVID-19 patients demonstrated zinc deficiency. This can be corrected with HDIVZn. Such treatment appears safe, feasible, and only associated with minimal peripheral infusion site irritation. This pilot study justifies further investigation of this treatment in COVID-19 patients.

KEYWORDS
COVID-19, randomized controlled trial, trial protocol, zinc

Abbreviations: HDIVZn, high dose intravenous zinc; HFNC, high-flow nasal cannula; NIV, noninvasive ventilation.

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1 | INTRODUCTION

Cheap, safe, and easily administered interventions to improve outcomes associated with COVID-19 infection remain an important goal. Zinc and Zn-salts inhibit viral infections in clinical and experimental settings,[1–10] including replication of SARS-coronavirus (SARS-CoV).[8] Thus, zinc supplementation might be beneficial in patients with COVID-19.[11–17] Clinical trials have been registered to test the efficacy of zinc against COVID, mostly with oral zinc supplementation.[18] However, the bioavailability of enterally administered zinc is low.[15] Thus, logically, intravenous zinc at a high dose should be the preferred mode of administration.[15] Unfortunately, no studies have assessed the feasibility, safety, and impact on zinc levels of high-dose intravenous zinc (HDIVZn). We designed a Phase IIa pilot double-blind randomized controlled trial (RCT) to determine the feasibility, safety, and biological efficacy of HDIVZn in subjects with COVID-19.

2 | METHODS

2.1 | Human ethics

The Austin Health Human Research Ethics Committee approved this study (HREC/62996/Austin-2020-208572(v3) and the trial was prospectively registered (Australia New Zealand Clinical Trial Register Registration no. ACTRN12620000454976).

2.2 | Study protocol and drug

A detailed clinical trial protocol has been published previously.[19] Briefly, we randomized consenting COVID-19 confirmed hospitalized adults with oxygen saturation (SpO_2) of 94% or less while on ambient air to either daily HDIVZn or saline placebo. Colorless pharmaceutical grade Zinc Chloride ($ZnCl_2$) stock solution obtained from Phebra Pty Ltd was diluted in 250 ml of normal saline and infused via

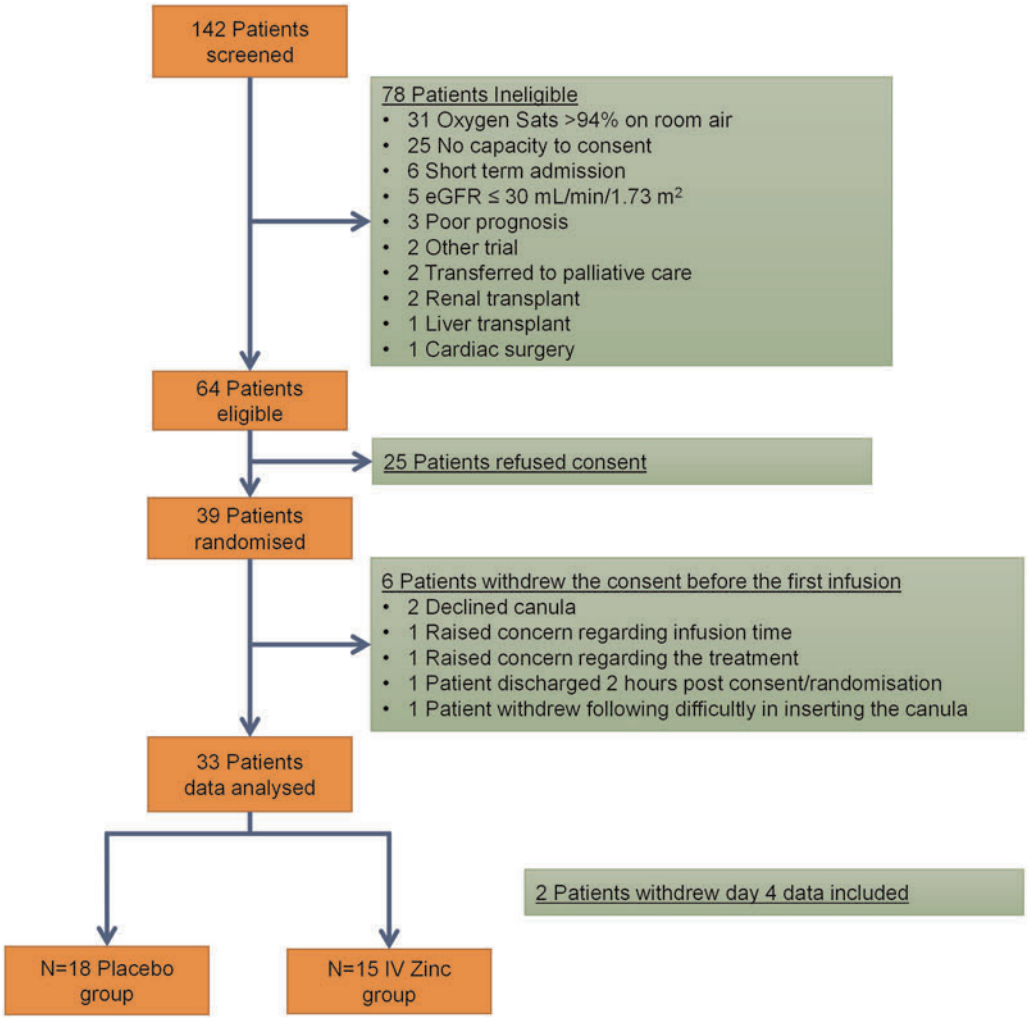


FIGURE 1 Flow chart showing patient recruitment and randomisation

peripheral intravenous access over 3 h at a dose of 0.5 mg/kg/day (elemental zinc concentration, 0.24 mg/kg/day) for a maximum of 7 days, or until hospital discharge or death. The investigators, study coordinators, treating physicians, bedside nurses, and patients/family remained blinded to the allocated study solution.

The serum trace metal analysis was carried out by Austin Pathology using inductively coupled plasma mass spectrometry (ICP-MS).

2.3 | Statistical analysis

Continuous variables are presented as means ± standard deviation and compared between groups with the Wilcoxon rank-sum test. Categorical variables are presented as numbers and percentages and compared with Fisher's exact tests. Variables are presented over time in line plots and compared between groups with a mixed-effect generalized linear model with Gaussian distribution, considering the patients as a random effect to account for repeated measurements, and with the time of measurement (as a continuous variable), group of randomization and an interaction between time x group as fixed effects. A two-sided value of $p < .05$ was taken to indicate statistical significance.

3 | RESULTS

We randomized 33 adults to either HDIVZn ($n = 15$) or placebo ($n = 18$; Figure 1 and Table 1). Hypertension, diabetes mellitus, and chronic cardiovascular disease were frequently present. We observed no drug-related severe adverse events during 94 administrations of HDIVZn However, three HDIVZn patients reported infusion site irritation, two on Day 4, and one on Day 6.

Mean serum zinc on Day 1 in the placebo and the HDIVZn group was 6.9 ± 1.1 and 7.7 ± 1.6 μmol/l, respectively, consistent with zinc deficiency. HDIVZn increased serum zinc levels above the deficiency cutoff of 10.7 μmol/l ($p < .001$) on Day 6. In contrast, serum zinc levels with placebo remained below this value (Figure 2A). In contrast, serum copper, calcium, and magnesium were within the normal range at baseline and were not affected by HDIVZn. As most of the patients enrolled in our study were donating blood across multiple trials, we had limited ability to collect enough blood samples to perform all the planned

TABLE 1 Demographics and baseline disease characteristics

Baseline Characteristics recorded on day 1 before receiving the first injection	HDIVZn ($n = 15$)	Control ($n = 18$)	p
Age, years (range)	59.8 ± 16.8 (25–84)	63.8 ± 16.9 (31–95)	.550
Male gender, *N* (%)	11 (73.3)	10 (55.5)	.711
Weight, kg (range)	86.6 ± 23.1 (50–128)	73.9 ± 16.8 (48 –100)	.073
Co-existing disorders, *N* (%)			
Hypertension	7 (46.7)	9 (50.0)	.999
Diabetes	3 (20.0)	3 (16.7)	.999
Chronic cardiovascular disease	4 (26.7)	3 (16.7)	.786
Chronic respiratory disease	0 (0.0)	2 (11.1)	.549
Cirrhosis	0 (0.0)	3 (16.7)	.294
Hepatic failure	0 (0.0)	1 (5.6)	.999
Drugs on Day 1, *N* (%)			
Dexamethasone	12 (80.0)	13 (72.2)	.911
Remdesivir	5 (33.3)	5 (27.8)	.999
Laboratory tests			
Zinc, μmol/l (range)	7.7 ± 1.6 (4.8–10.5)	6.9 ± 1.1 (4.6–8.5)	.122
Creatinine, μmol/l (range)	71.4 ± 24.9 (37–120)	80.4 ± 33.7 (37–156)	.718
C-reactive protein, mg/l (range)	123.7 ± 72.7 (10–223)	91.4 ± 75 (4–294)	.126
Troponin I, ng/l (range)	91.2 ± 223.2 (4–786)	18.1 ± 21.2 (2–71)	.981
Lactate, mmol/l (range)	0.661 ± 2.1 (0.8–2.9)	0.634 ± 2.4 (0.7–3.1)	.868
White blood cell count, $\times 10^3$ cells/mm^3 (range)	5.5 ± 2.7 (2.2–10.7)	6.6 ± 3.1 (2.4–12.7)	.193

Note: Values are mean ± *SD* or percentages as indicated; range indicates Min and Max values.

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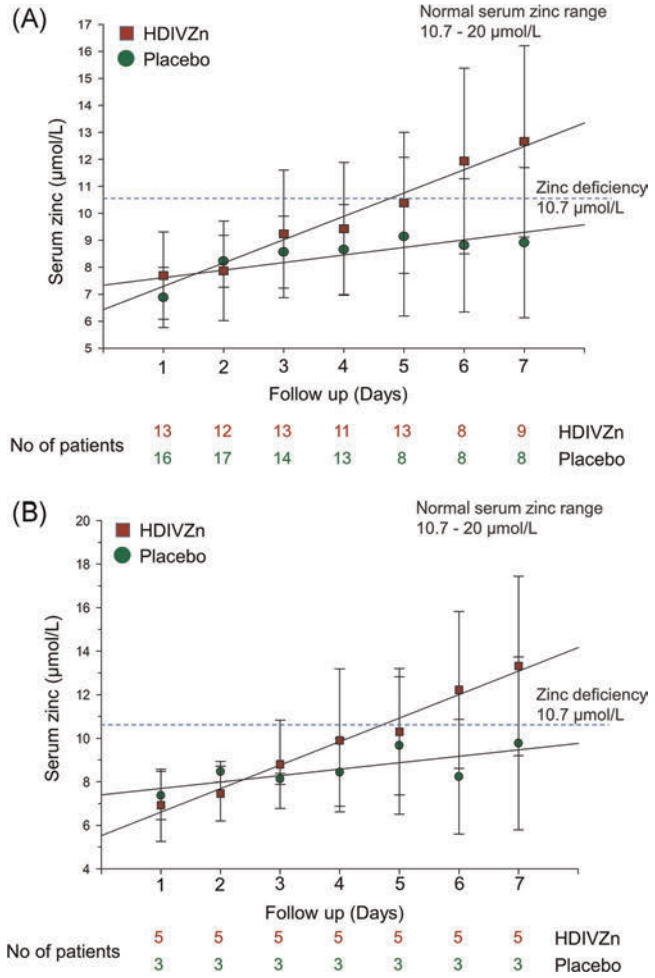


FIGURE 2 High dose intravenous zinc (HDIVZn) increased serum zinc levels in zinc-deficient COVID-19 patients

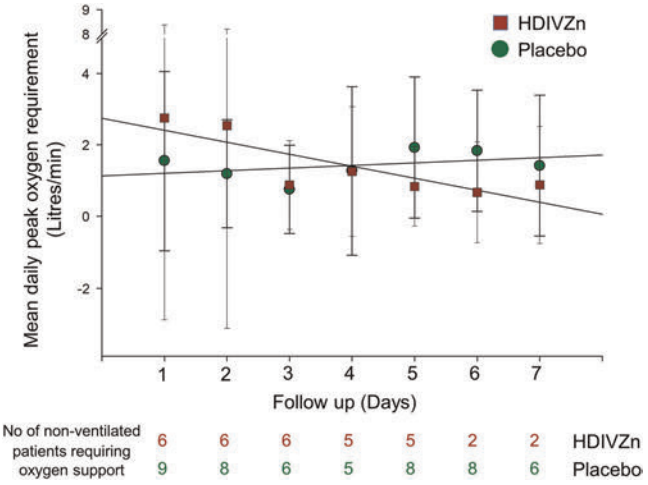


FIGURE 3 The effect of high dose intravenous zinc (HDIVZn) on mean daily peak oxygenation requirement in COVID-19 patients

biochemical tests daily, including the serum zinc levels. Figure 2B shows the serum zinc for eight patients who had their serum zinc determined at all the time points across the 7-day study period.

The primary outcome in non-ventilated patients was assessed as the level of oxygenation expressed as oxygen flow (in liters/min) required to maintain blood oxygen levels (SpO_2) above 94% and the worst (lowest) PaO_2/FiO_2 ratio in ventilated patients. Our study did not reach its target enrollment because stringent public health measures markedly reduced new patient presentations to zero. Consequently, we could not adequately assess the primary outcome of whether HDIVZn reduced the level of oxygenation in non-ventilated (Figure 3) or improved the PaO_2/FiO_2 ratio in the four ventilated patients (data not shown) and other clinical efficacy outcomes (Table 2).

4 | DISCUSSION

Zinc therapy is logical in COVID-19 because of the inhibitory effect of zinc on viral replication and because low zinc levels appear common in COVID-19 patients.[20,21] In a recently published study among a cohort of 47 COVID-19 patients, 27 (57.4%) were found to be zinc deficient.[20] In a Japanese study among a cohort of 29 COVID-19 patients, 9 (31%) were found to be zinc deficient.[21]

There is no globally accepted cutoff level for zinc deficiency. However, Hotz et al.[22] based on the data from the second National Health and Nutrition Examination Survey (NHANES II) suggested that for males greater than 10 years of age serum zinc levels below 61–74 µg/dl (9.3–11.3 µmol/l) depending on the fasting status can be considered as a cutoff value for zinc deficiency. Furthermore, the International Zinc Nutrition Consultative Group (IZiNCG)[23] has defined 10.7 µmol/l as a cutoff value for zinc deficiency, and this value was previously used in an Australian study.[24] Serum zinc levels less than 60 µg/dl (9.1 µmol/l) are defined as clinical zinc deficiency in Japan. However, in a recently published Japanese study, the zinc deficiency cutoff value was set at <70 µg/dl (10.7 µmol/l) to enable direct comparison with domestic and international information.[21]

A previous study has determined that the mean serum zinc in the Australian population is 13 ± 2.4 and 13 ± 2.5 µmol/l for men and women, respectively.[24] One of the leading causes of zinc deficiency is aging; however, in a well-characterized cohort of healthy Australians with an average age of 70.6 ± 7 years, the serum zinc levels were 12.7 ± 2.5 µmol/l.[25] In our study, 100% of patients (33/33) were zinc deficient as the mean serum zinc values in the placebo, and the HDIVZn group were 6.9 ± 1.1 and 7.7 ± 1.6 µmol/l, respectively, well below the zinc deficiency cutoff value of 10.7 µmol/l. Therefore, our results suggest that such zinc deficiency seen in COVID-19 patients may be an acute phase reaction, specifically due to COVID-19 infection.[26]

Zinc deficiency has been associated with a twofold increase in complication rates,[20] greater risk of acute respiratory distress syndrome, longer hospital stay, and increased mortality[20] with an independent relationship between low serum zinc level and disease severity.[21] A recent European study with 35 participants confirmed that most COVID-19 patients were zinc deficient with lower zinc

FOI 26-3132 - Document 5

TABLE 2 Clinical outcomes

Comparison of ordinal scale	HDIVZn ($n = 15$)	Control ($n = 18$)
Eight-level ordinal scale at Day 1, *N* (%)		
3. Hospitalized, no supplemental oxygen	6 (40.0)	9 (50.0)
4. Hospitalized, with supplemental oxygen	5 (33.3)	8 (44.4)
5. Hospitalized, NIV, and/or HFNC	1 (6.7)	1 (5.6)
6. Hospitalized, mechanical ventilation	3 (20.0)	0 (0.0)
Eight-level ordinal scale at Day 7, *N* (%)		
2. Not hospitalized, with limitations	2 (15.4)	6 (33.3)
3. Hospitalized, no supplemental oxygen	6 (46.2)	6 (33.3)
4. Hospitalized, with supplemental oxygen	2 (15.4)	5 (27.8)
5. Hospitalized, NIV, and/or HFNC	0 (0.0)	1 (5.6)
6. Hospitalized, mechanical ventilation	2 (15.4)	0 (0.0)
8. Death	1 (7.7)	0 (0.0)
Eight-level ordinal scale at Day 14, *N* (%)		
0. Not hospitalized, no infection	2 (14.3)	2 (11.1)
2. Not hospitalized, with limitations	5 (35.7)	10 (55.6)
3. Hospitalized, no supplemental oxygen	3 (21.4)	2 (11.1)
4. Hospitalized, with supplemental oxygen	3 (21.4)	0 (0.0)
5. Hospitalized, NIV, and/or HFNC	0 (0.0)	1 (5.6)
8. Death	1 (7.1)	3 (16.7)
Eight-level ordinal scale at Day 28, *N* (%)		
0. Not hospitalized, no infection	2 (14.3)	2 (11.1)
1. Not hospitalized, without limitation	1 (7.1)	0 (0.0)
2. Not hospitalized, with limitations	7 (50.0)	12 (66.7)
3. Hospitalized, no supplemental oxygen	1 (7.1)	1 (5.6)
4. Hospitalized, with supplemental oxygen	1 (7.1)	0 (0.0)
8. Death	2 (14.3)	3 (16.7)

Abbreviations: HFNC, high-flow nasal cannula; NIV, noninvasive ventilation.

levels in non-survivors.[27] Given the above observations, various investigators have hypothesized that zinc therapy may be beneficial in COVID-19,[11–13] but zinc bioavailability from enteral administration is low, and the low dosage is inadequate.[15]

A recently published study with four participants suggested that oral administration of elemental zinc to a maximum amount not exceeding 200 mg (zinc salt lozenges) was well tolerated and may have been associated with positive recovery from COVID-19.[28] In contrast, a single-center retrospective study of hospitalized patients with COVID-19 ($n = 242$) determined a lack of association between zinc sulfate intake (100 mg elemental zinc daily) and survival. Hydroxychloroquine (HCQ) has been shown to act as a zinc ionophore, increasing zinc uptake and inducing apoptosis in malignant cells.[13,29] A recent trial by Boulware et al.[29] determined that zinc, in combination with HCQ, was ineffective as there was no evidence that HCQ, in combination with zinc, reduced the incidence of COVID-19 after a high-risk exposure. However, the authors conceded that the study could not be considered conclusive. It was limited by the small sample size of the zinc cohort exacerbated by the lack of details regarding zinc formulation, dose, and treatment duration.

In contrast, Derwand et al.[30] showed that oral supplementation with 50 mg elemental zinc (zinc sulfate), in combination with HCQ (200 mg twice a day), and azithromycin (500 mg per day) for five consecutive days was associated with significantly fewer hospitalizations. Similarly, Carlucci et al.,[31] using the same combination determined that 50 mg elemental zinc (zinc sulfate), when added to HCQ and azithromycin, decreased the mortality and hospitalization. Several shortcomings of these studies include the fact that interventions were not controlled nor blinded. Patients were not randomized or monitored for various other laboratory parameters. However, the most significant limitation of the studies mentioned above is the lack of data showing whether oral zinc supplementation on its own or in combination with HCQ indeed improved the serum zinc status among COVID-19 patients.

The upper daily oral intake limit for elemental zinc is 40 mg/day as defined by the National Institutes of Health and a lower 25 mg/day as defined by the European Food Safety Authority. A recent study noted that enteral delivery (via a nasogastric feeding tube) of 100–150 mg/day elemental zinc supplied as zinc acetate (brand name Nobelzin) takes between 14 and 24 days to correct zinc deficiency, defined as serum zinc less than 70 μg/dl (equivalent to 10.7 μmol/l) in hospitalized Japanese COVID-19 patients.[21] In comparison, our HDIVZn regimen using an elemental zinc dose ranging from a minimum daily dose of 12 mg (in a 50 kg patient) to a maximum of 30.7 mg (in a 128 kg patient) corrected zinc deficiency within a week without any toxicity or side effects. The need to use significantly higher doses of zinc than the recommended upper daily oral intake stems from an analysis by Hambidge et al.,[32] indicating that the maximal absorption (A_{max}) for zinc via oral delivery is only ≈7 mg Zn/day. This low A_{max} is due to the molecular complexities of oral zinc absorption into and across the enterocyte and basolateral membrane. Overall, our study suggests that intravenous zinc overcomes low A_{max} of oral zinc supplementation. Furthermore, intravenous zinc may be superior to oral zinc supplementation in rectifying the acute phase zinc deficiency seen in COVID-19 patients; however, a direct comparative study (intravenous vs. oral Zn) is warranted.

In summary, our study provides the first evidence showing the safety and feasibility of intravenous zinc treatment and the ability of HDIVZn to reverse the acute phase zinc deficiency associated with COVID-19. These findings support further investigation of this treatment in larger RCTs.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

FOI 26-3132 - Document 5

AUTHOR CONTRIBUTIONS

Oneel Patel, Rinaldo Bellomo, and Joseph Ischia designed and directed the project. Christine McDonald, Daryl Jones, and Damien Bolton contributed to the design and implementation of the trial protocol. Vidyasagar Chinni, John El-Khoury, Marlon Perera, and Jason Trubiano collected the data. Ary S. Neto performed the statistical analysis. Emily See developed the data collection database. Oneel Patel, Rinaldo Bellomo, and Joseph Ischia wrote the manuscript. All authors discussed the results, edited, and commented on the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/jmv.26895.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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How to cite this article: Patel O, Chinni V, El-Khoury J, et al. A pilot double-blind safety and feasibility randomized controlled trial of high-dose intravenous zinc in hospitalized COVID-19 patients. *J Med Virol*. 2021;93:3261-3267. https://doi.org/10.1002/jmv.26895

FOI 26-3132 - Document 5

Life Sciences 254 (2020) 117775



Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie



A systematic review on use of aminoquinolines for the therapeutic management of COVID-19: Efficacy, safety and clinical trials



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ARTICLE INFO

Keywords:
COVID-19
Aminoquinolines
Chloroquine
Hydroxychloroquine
Safety
Anti-viral mechanism

ABSTRACT

Recent global outbreak of the pandemic caused by coronavirus (COVID-19) emphasizes the urgent need for novel antiviral therapeutics. It can be supplemented by utilization of efficient and validated drug discovery approaches such as drug repurposing/repositioning. The well reported and clinically used anti-malarial aminoquinoline drugs (chloroquine and hydroxychloroquine) have shown potential to be repurposed to control the present pandemic by inhibition of COVID-19. The review elaborates the mechanism of action, safety (side effects, adverse effects, toxicity) and details of clinical trials for chloroquine and hydroxychloroquine to benefit the clinicians, medicinal chemist, pharmacologist actively involved in controlling the pandemic and to provide therapeutics for the treatment of COVID-19 infection.

1. Introduction

Coronavirus disease 2019 (COVID-19 or 2019-nCoV) continues to spread all over the world. The infection has spread over to 213 countries (23,14,621 confirmed cases and 157,847 confirmed deaths) since its outbreak in November 2019 in China (as on 20 April 2020) (Fig. 1) [1–5]. The worldwide pandemic and uncontrolled scenario demands use of efficient drug discovery approaches such as computational chemistry and biology, high throughput screening (HTS), artificial intelligence (AI), drug repurposing etc. for effective control [6–12]. Among these approaches, drug repurposing (or drug repositioning) has been implemented for anti-viral drug discovery (Fig. 2) [13–90]. It has helped to conduct in vitro studies and clinical trials for a dozen of chemical molecules and evaluate their anti-viral efficacy against COVID-19 (Table 1) [91–93].

One of the examples of successful application of drug discovery approaches is drug repurposing of the traditional anti-malarial drugs aminoquinolines namely, chloroquine (CQ) and hydroxychloroquine (HCQ) (Fig. 3). Both are synthetic anti-malarial drugs with rapid absorption. Chloroquine and hydroxychloroquine are water soluble; the latter is more soluble due to presence of hydroxyl group and possesses plasma half-life of 900 h and 1300 h, respectively [94]. During chronic treatment the drugs gets accumulated in tissues [95]. The selected anti-malarial drugs have been used for last 70 years. They are economic, have proven safety profile and are categorized as essential medicines by World Health Organization (WHO) [96]. Aminoquinolines have effectively reduced viral replication in Zika virus, Chikungunya virus, SARS-associated coronavirus (CoV) and MERS-CoV [97–100]. Chloroquine and hydroxychloroquine has shown inhibition of SARS-CoV-2 replication [101]. Clinical trials have demonstrated the effective role of chloroquine phosphate (dose 500 mg/day) against COVID-19 [102]. The N-hydroxyethyl substituted derivative of chloroquine, hydroxychloroquine is less toxic, more soluble and has similar activity towards COVID-19 inhibition. There is continuous requirement to explore the molecular mechanism towards underlying antiviral action and clinical benefits of aminoquinolines and the toxicity profile. The detailed outcomes will help to design the randomized clinical trials [95,103–107]. The present manuscript provides a systematic review of mechanism of action, efficacy, and safety of chloroquine and hydroxychloroquine which are being used as therapeutic measure to cure COVID-19 infection.

2. Mechanism of action

For viral replication, a stable acidic pH of endosomes, lysosomes, Golgi complex of host is required. The bioaccumulation properties of both the selected aimnoquinolines explain the antiviral mechanism of their action [108]. Chloroquine increases the pH of intracellular vacuoles. In lysosomes, it alters the catalysis of the protein degradation pathways through acidic hydrolases. It also alters endosomal

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https://doi.org/10.1016/j.lfs.2020.117775
Received 21 April 2020; Accepted 7 May 2020
Available online 11 May 2020
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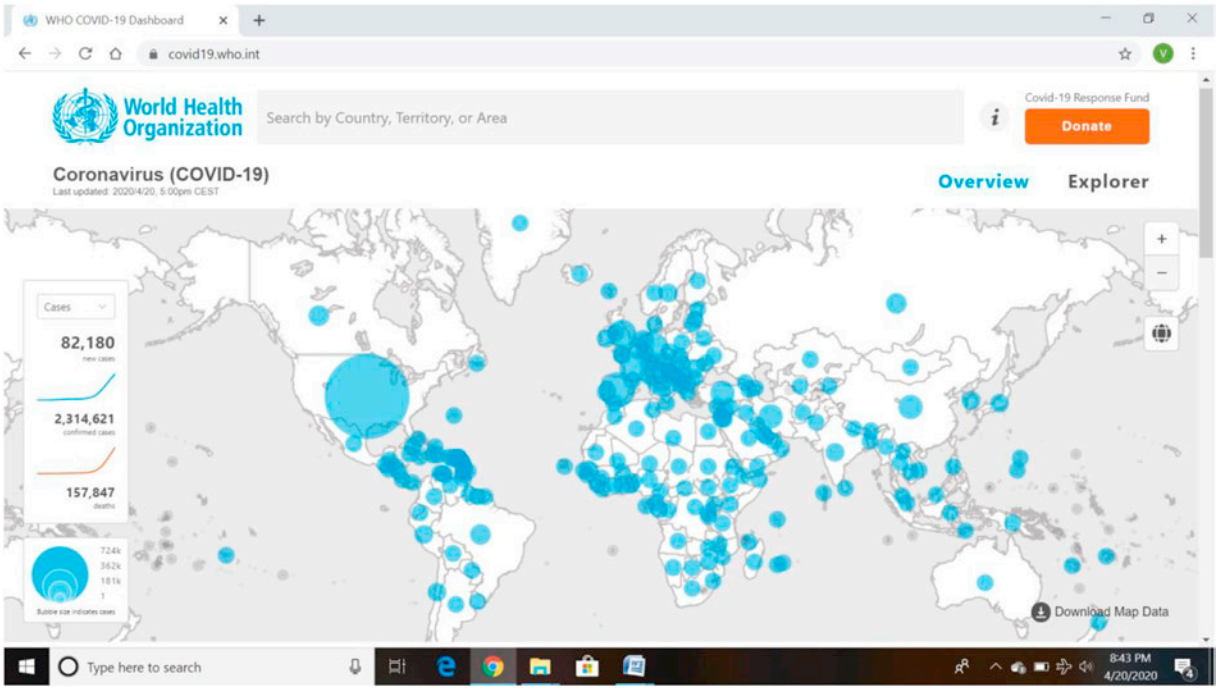


Fig. 1. Global COVID-19 spread showing number of confirmed cases (blue color) (as on 20 April 2020, 8:43 pm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

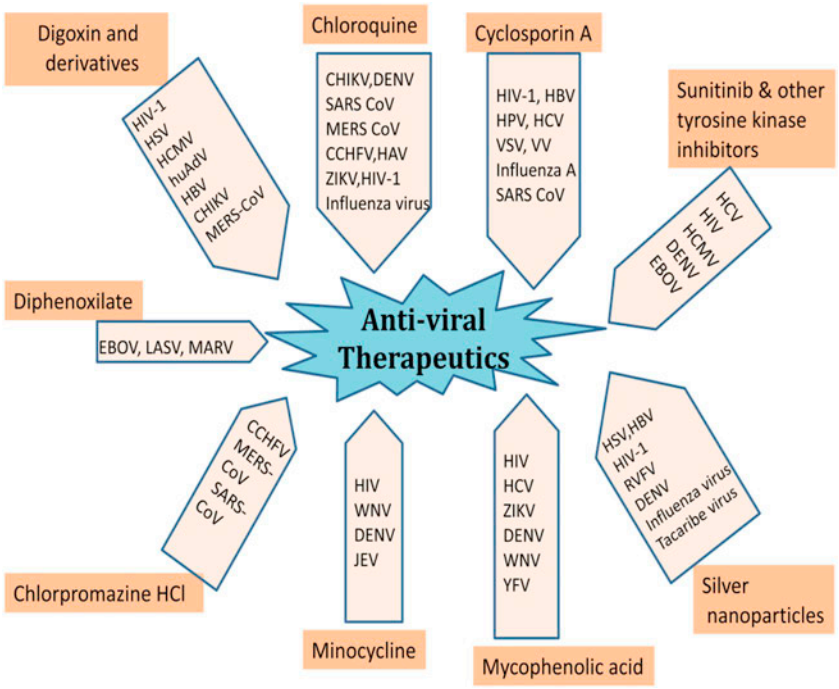


Fig. 2. Examples of drug repurposing for viral inhibition [13–90].

macromolecule synthesis and in Golgi apparatus it affects post-translational modifications. The antirheumatic response is produced by interfering with the immunological process which occurs in macrophages and antigen-presenting cells [109]. The mechanism involved for antiviral action is similar. It decreases the pH and interferes with the viral fusion process. In coronavirus, chloroquine binds to the cellular receptors and changes the glycosylation [110]. Chloroquine possesses selective and reversible immunomodulatory effect through its action on human $CD4^+$ T-cells which is mediated by inhibition of JNK catalytic activity [109].

Hydroxychloroquine exerts similar mechanism of antiviral action and some of the key features are – i) increases the pH, ii) modulation of activated immune cells, iii) downregulation of expression of Toll-like receptors (TLR), iv) downregulation of TLR-mediated signal transduction; v) interleukin-6 formation drops; vi) reduces the formation of pro-inflammatory cytokines and other mediators to control inflammation [105,110,111].

The recent literature review helps to propose the possible mechanism of action of chloroquine and hydroxychloroquine through three ways i.e. immunomodulatory effect, zinc ionophore effect and

Table 1
Clinical trials details for studies going on for use of chloroquine (CQ) and hydroxychloroquine (HCQ) in the treatment of COVID-19 infection [5].

ID	Country	Number of patients	Intervention
NCT04303507	USA	40,000	Drug: CQ or HCQ A loading dose of 10 mg base/kg followed by 155 mg daily (250 mg CQ phosphate salt or 200 mg of or HCQ sulphate) will be taken for 3 months drug: CQ or HCQ A loading dose of 10 mg base/kg followed by 155 mg daily (250 mg CQ phosphate salt or 200 mg of or HCQ sulphate) will be taken for 3 months
NCT04335084	ProgenBiome, US	600	HCQ, vitamin C, vitamin D and zinc (through dietary supplement)
NCT04330586	–	141	Ciclesonide metered dose inhaler; HCQ
NCT04342169	University of Utah, US	400	HCQ, placebo oral tablet
NCT04328012	USA	4000	Lopinavir, HCQ sulfate, losartan, placebos
NCT04333732	Washington University School of Medicine (USA, Australia, Canada, Ireland, South Africa, UK)	55,000	Low dose: CQ/HCQ, mid dose CQ/HCQ, high dose CQ/HCQ, placebo
NCT04343677	11 MDG, US	1450	HCQ, dietary supplement, placebo
NCT04334967	Providence Medical Group Infectious Disease, US	1250	HCQ, dietary supplement of vitamin C
NCT04333225	Baylor Health Care System, US	360	HCQ
NCT04328961	University of Washington, US	2000	HCQ sulfate, ascorbic acid
NCT04318444	Columbia University Irving Medical Centre, US	1600	HCQ, placebo oral tablet
NCT04329832	Intermountain Health Care Inc. US	300	HCQ, azithromycin
NCT04334382	Intermountain Health Care Inc. US	1550	HCQ, azithromycin
NCT04332991	Massachusetts General Hospital, US	510	HCQ, placebo
NCT04328467	University of Minnoesota, US	3500	HCQ, placebo
NCT04336332	Rutgers, The State University of New Jersey, US	160	Combination product HCQ sulfate + azithromycin; drug: HCQ sulfate
ACTRN12620000447954	Australia	150	HCQ is not considered a trial intervention
ACTRN12620000447987	Australia	680	CQ phosphate (tablet, 500 mg, oral) for 10 week trial period followed with plasma CQ levels
NCT04328493	Vietnam	240	CQ will be administered orally, as tablets. For unconscious patients CQ can be crushed and administered as a suspension via a nasogastric tube. A loading dose of 1200 mg CQ phosphate base, administered with food where possible, is given on the first 24 h after randomization. Following, patients will receive a dose of CQ phosphate base of 300 mg once daily for 9 days (unless they are <60 kg, when the dose will be reduced following its pharmacokinetic properties). The total duration of treatment with Chloroquine will be 10 days
NCT04342650	Brazil	210	CQ diphosphate, placebo oral tablet
NCT04329572	Azidus Brasil	400	HCQ sulfate, azithromycin tablets
NCT04321278	Hospital Israelita Albert Einstein, Brazil	440	HCQ + azithromycin, HCQ
NCT04322123	Hospital do Coracao. Brazil	630	HCQ oral product, HCQ _ azithromycin
NCT04333628	HaEmek Medical Center, Israel	210	CQ, standard care
NCT04303507	–	40,000	CQ or HCQ, placebo
EUCTR2020-001345-38-GR	Uni-Pharma Kleon Tsetis Pharmaceutical Lab SA, Greece	60	UNIKINON tablets 200 mg, CQ phosphate
NCT04321993	Canada	1000	Lopinavir/ritonavir, HCQ sulfate, baricitinib, sarilumab
NCT04324463	Population Health Research Institute, Canada	1500	Azithromycin, HCQ
NCT04329611	University of Calgary, Canada	1660	HCQ
NL8490	the Netherlands	950	Standard supportive care, CQ arm (loading dose 600 mg as CQ base followed by 300 mg 12 h later followed by 300 mg twice a day; total treatment duration: 5 days); HCQ arm (loading dose 400 mg twice daily followed by 200 mg twice a day; total treatment duration 5 days); no antiviral treatment arm
NCT04322396	Denmark	226	Azithromycin, HCQ, placebo oral tablet
NCT04334928	Plan Nacional sobre el Side, Spain	4000	Drug: emitricitabine/tenofovir disoproxil, drug: HCQ, drug: placebo: emtricitabine/tenofovir disoproxil placebo; drug: placebo: HCQ
EUCTR2020-001385-11-ES	Spain	4000	HCQ
EUCTR2020-001565-37-ES	ISGlobal, Spain	440	HCQ sulfate, placebo (oral use)
EUCTR2020-001421-31-ES	Delos Clinical, Spain	1530	HCQ sulfate
NCT04332094	Spain	276	Tocilizumab, HCQ, azithromycin
NCT04331834	Barcelona Institute for Global Health, Spain	440	HCQ, placebos
EUCTR2020-001366-11-ES	FIB-HCSC, Spain	1,00,000	Remedesivir, CQ, HCQ sulfate, lopinavir/ritonavir, interferon b 1A
IRCT20100228003449N29	Iran	50	HCQ 400 mg single dose + Oseltamivir 75 mg twice daily + lopinavir/ritonavir 200/50 mg two tablets twice daily for 5 days, sofosbuvir/ledipsavir 400/100 mg daily for 10 days,

(*continued on next page*)

Table 1 (*continued*)

ID	Country	Number of patients	Intervention
IRCT20100228003449N28	Tehran University of Medical Sciences, Iran	30	Intervention 1: concomitant with the national corona treatment recommendation (HCQ + Oseltamivir + Lopinavir/ritonavir), patients will receive interferon B, sub type 1b with dose of 250 μg subcutaneously every other day for 14 days. Intervention 2: control group will receive the national corona treatment recommendation (HCQ + oseltamivir + lopinavir/ritonavir) for at least 5 days
IRCT20100228003449N29	Tehran University of Medical Sciences, Iran	50	Intervention group: tab HCQ 400 mg single dose + cap oseltamivir 75 mg, twice daily + tab lopinavir/ritonavir 200/50 mg two tablets twice daily for at least 5 days and one tablet of Sofosbuvir/ledipasvir 400/100 mg daily for 10 days. Intervention 2 (control group): tab HCQ 400 mg single dose + cap oseltamivir 75 mg, twice daily + tab lopinavir/ritonavir 200/50 mg two tablets twice daily for at least 5 days
NCT04331470	Iran	30	
IRCT20151227025726N12	Shahid Beheshti Unmversity of Medical Sciences, Iran	20	Tab HCQ 400 mg P.O. twice daily for 5 days + tab oseltamivir 75 mg P.O. twice daily for 5 days + tab lopinavir/ritonavir 200/50 mg P.O. two tablets twice daily for 5 days + interferon beta-1a 44 mg every other day S.C. for 10 days
IRCT20100228003449N27	Tehran University of Medical Sciences, Iran	30	Intervention group 1: concomitant with the national corona treatment recommendation (HCQ + oseltamivir + lopinavir/ritonavir), patients will receive Interferon beta-1b with dose of 250 μg subcutaneously every other day for 14 days Intervention 2 (control group): control group will receive the national corona treatment recommendation (HCQ + oseltamivir + lopinavir/ritonavir) for at least 5 days
NCT04343768	Shahid Beheshti University of Medical Sciences	60	HCQ, lopinavir/ritonavir, interferon beta-1A, interferon Beta-1B
NCT04343092	–	50	Ivermectine, HCQ sulfate, placebos
NCT04318015	National Institute of Respiratory Diseases-Mexico	400	HCQ
NCT04340349	Mexico	100	HCQ sulfate, bromhexine (8 mg)
NCT04342221	University Hospital Tubingen, Germany	220	HCQ sulfate, placebo
NCT04340544	Germany	2700	HCQ, placebo
EUCTR2020-000936-23-FR	INSERM, France, Belgium, Luxembourg, Netherlands, Germany, UK, Spain	3100	Lopinavir/ritonavir, HCQ
EUCTR2020-001010-38-NO	Akershus University Hospital, Norway	200	HCQ sulfate
NCT04316377	Akershus University Hospital, Norway	202	HCQ sulfate
ACTRN12620000457943	New Zealand	70	Oral administration of HCQ capsules for 5 days. Day 1- 800 mg (4 capsules) HCQ stat day 2 to 5- 400 mg (2 capsules)
NCT04323631	Rambam MC	1116	HCQ, control group will not receive HCQ
NCT04333654	Sanofi – US, France	210	HCQ SAR321068, placebo
EUCTR2020-001281-11-FR	URCIP-CHU Saint Etienne, France	50	HCQ sulfate
EUCTR2020-001435-27-FR	Centre Hospitalier Universitaire de Bordeaux, Etablissement Public, France	1057	HCQ (200 mg), imatinib (400 mg), favipiravir
EUCTR2020-001281-11-FR	France	50	HCQ
NCT04315948	Hospital Civils de Lyon, France	3100	Remdesivir, lopinavir/ritonavir, interferon beta 1A, HCQ, standard of care
NCT04325893	France, Monaco	1300	HCQ, placebo
NCT04315896	National Institute of Respiratory Diseases, Mexico	500	HCQ, placebo oral tablet
NCT04318015	National Institute of Respiratory Diseases, Mexico	400	HCQ, placebo oral tablet
NCT04341493	Hospital Materno-Perinatal, Mexico	86	Nitazoxanide (500 mg), HCQ
JPRN-jRCTs031190227	Gunma University Hospital, Japan	50	Lopinavir, ritonavir, HCQ with or without oseltamivir (oral)
NCT04328272	Khyber Medical University, Peshawar, Pakistan	75	Drug: HCQ (200 mg oral tablet), drug: azithromycin (500 mg oral tablet), dietary supplement: glucose tablet
NCT04338698	University of Health Sciences, Lahore	500	HCQ, oseltamivir, azithromycin
ChiCTR2000031454	The Fifth Affiliated Hospital of Sun Yat-Sen University, China	Experimental group: 28; Control group: 28	Experimental group: rabeprazole + CQ Control group: lopinavir + rabeprazole
ChiCTR2000030417	Harbin Peiyou Jiandi Biotechnology Co Ltd., China	Experimental group: 15; Control group: 15	Experimental group: combined standard therapy of CQ phosphate aerosol inhalation solution Control group: water for injection atomization inhalation combined with standard therapy
ChiCTR2000030054	Zhongshan Hospital Affiliated to Xiamen University, China	HCQ sulfate group: 40 CQ Phosphate Group: 40 Control group: 20	HCQ sulphate group: HCQ sulfate 0.2 g twice daily for 14 days CQ phosphate group: day 1 and 2 – CQ phosphate 1 g; day 3 to 12 – CQ phosphate 0.5 g Control group: recommended treatment plan for novel coronavirus pneumonia diagnosis and treatment plan
ChiCTR2000030031	The Sixth Affiliated Hospital of Guangzhou Medical University, China	Phosphoric chloroquine: 80; Placebo: 40	Phosphoric chloroquine: two tablets twice daily + recommended therapy Placebo: 2 tablets placebo twice daily + recommended therapy

(*continued on next page*)

Table 1 (*continued*)

ID	Country	Number of patients	Intervention
ChiCTR2000029992	Zhongshan Hospital Affiliated to Xiamen University, China	CQ phosphate group: 40 HCQ sulfate group: 40 Routine treatment group: 20	CQ phosphate group: Day 1 and 2 – CQ phosphate 1 g Day 3 to 12 – CQ phosphate 0.5 g HCQ sulfate group: HCQ sulfate 0.2 g twice daily for 14 days Routine treatment group: recommended treatment plan for novel coronavirus pneumonia severe and critical cases
ChiCTR2000029988	Zhongnan Hospital of Wuhan University, China	Experimental group: 40; Control group: 40	Experimental group: CQ phosphate Control group: no
ChiCTR2000029935	HwaMei Hospital, University of Chinese Academy of Sciences, China	Case series: 100	Conventional treatment combined with CQ phosphate
ChiCTR2000029899	Peking University Third Hospital, China	HCQ sulfate: 50; CQ phosphate: 50	HCQ sulfate: day 1- first dose of 6 tablets (0.1 g/tablet); second dose after 6 h of 6 tablets (0.1 g/tablet) Day 2 to 5- two tablets (0.1 g/tablet) twice daily CQ phosphate: day 1 to 3–500 mg twice daily; day 4 to 5–250 mg twice daily
ChiCTR2000029898	Peking University Third Hospital, China	HCQ sulfate: 50; CQ phosphate: 50	HCQ sulfate: day 1- first dose of 6 tablets (0.1 g/tablet); second dose after 6 h of 6 tablets (0.1 g/tablet) Day 2 to 5- two tablets (0.1 g/tablet) twice daily CQ phosphate: day 1 to 3–500 mg twice daily; day 4 to 5–250 mg twice daily
ChiCTR2000029868	Ruijin Hospital, Shanghai Jiaotong University School of Medicine, China	Experimental group: 180; Control group: 180	Experimental group: HCQ sulfate oral tablets; Control group: conventional treatment meet the Guideline
ChiCTR2000029837	Jingzhou Central Hospital, China	Phosphoric chloroquine: 80; Placebo: 40	Phosphoric chloroquine: 2 tablets twice daily + recommended therapy; Placebo: 2 tablets twice daily + recommended therapy
ChiCTR2000029826	Jinhzhou Central Hospital, China	Phosphoric chloroquine: 30; Placebo: 15	Phosophoric chloroquine: two tablets twice daily + recommended therapy Placebo: 2 tablets twice daily
ChiCTR2000029803	Renmin Hospital of Wuhan University, China	A1:80; A2:80; B1:80; B2:80	A1: HCQ, small dose A2: HCQ, high dose B1: abidol hydrochloride, small dose B2: abidol hydrochloride, high dose
ChiCTR2000030987	Beijing Chao-yang Hospital, China	Experimental group 1: 50; Experimental group 2: 50; Control group: 50	Experimental group 1: oral trial drug favipiravir tablet + CQ phosphate tablet Experimental group 2: oral trial drug favipiravir tablet Control group: oral placebo treatment
ChiCTR2000029762	The First affiliated Hospital of Chongqing Medical University, China	Experimental group: 30; Control group: 30	Experimental group: conventional treatment and HCQ Control group: conventional treatment
ChiCTR2000029760	Chongqing Medical University, China	Experimental group: 120; Control group: 120	Experimental group: HCQ Control group: lopinavir/ritonavir
ChiCTR2000029761	The First affiliated Hospital of Chongqing Medical University, China	Low-dose group: 60; Medium-dose group: 60; High-dose group: 60; Control group: 60	Low-dose group: HCQ low dose + conventional therapy Medium-dose group: HCQ medium dose + Conventional therapy High-dose group: HCQ high dose + conventional therapy Control group: conventional therapy
ChiCTR2000029741	The Fifth Affiliated Hospital Sun Yat-Sen University, China	Experimental group: 56; Control group: 56	Experimental group: CQ phosphate; control group: lopinavir/ritonavir
ChiCTR2000029740	The First Hospital of Peking University, China	HCQ group: 54; Control group: 24	HCQ group: Oral intake HCQ 0.2 twice a day; Control group: conventional therapy
ChiCTR2000030718	Zhongnan Hospital of Wuhan University, China	Experimental group: 40; Control group: 40	Experimental group: CQ phosphate Control group: none
ChiCTR2000029975	China	10	CQ phosphate dissolved in 5 ml of normal saline, q 12 h, inhaled by atomization for one week
ChiCTR2000029939	China	100	CQ phosphate, Standard treatment
ChiCTR2000029609	China	205	Mild-moderate CQ group: oral CQ phosphate; Mild-moderate combination group: CQ phosphate plus lopinavir/ritonavir; severe CQ group: oral CQ phosphate
ChiCTR2000029559	China	300	Group 1: HCQ 0.1 g oral twice a day Group 2: HCQ 0.2 g oral twice daily
ChiCTR2000029542	China	20	Oral CQ 0.5 g twice daily for 10 days
ChiCTR2000029826	China	45	Two tablets CQ phosphate twice daily Two tablets placebo twice daily
ChiCTR2000031204	Beijing Institute of Pharmacology and Toxicology, China	Treatment group: 150 Control group: 150	Treatment group: oral CQ phosphate tablets Control group: oral placebo group
NCT04342156	Tan Tock Seng Hospital	3000	HCQ sulfate 200 mg tablet
NCT04261517	China	30	HCQ
NCT04319900	China	150	Favipiravir tablets + CQ phosphate tablets, favipiravir tablets, placebo
NCT04307693	Korea	150	Lopinavir/ritonavir, HCQ sulfate
NCT04332835	Universidad del Rosario, Cambodia	80	Plasma, HCQ, azithromycin
NCT04338906	–	334	Camostat mesilate, placebo, HCQ
NCT04336748	–	440	HCQ

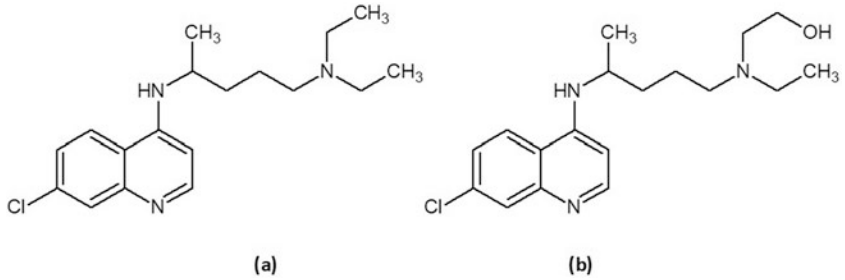


Fig. 3. Chemical structures of (a) Chloroquine and (b) Hydroxychloroquine.

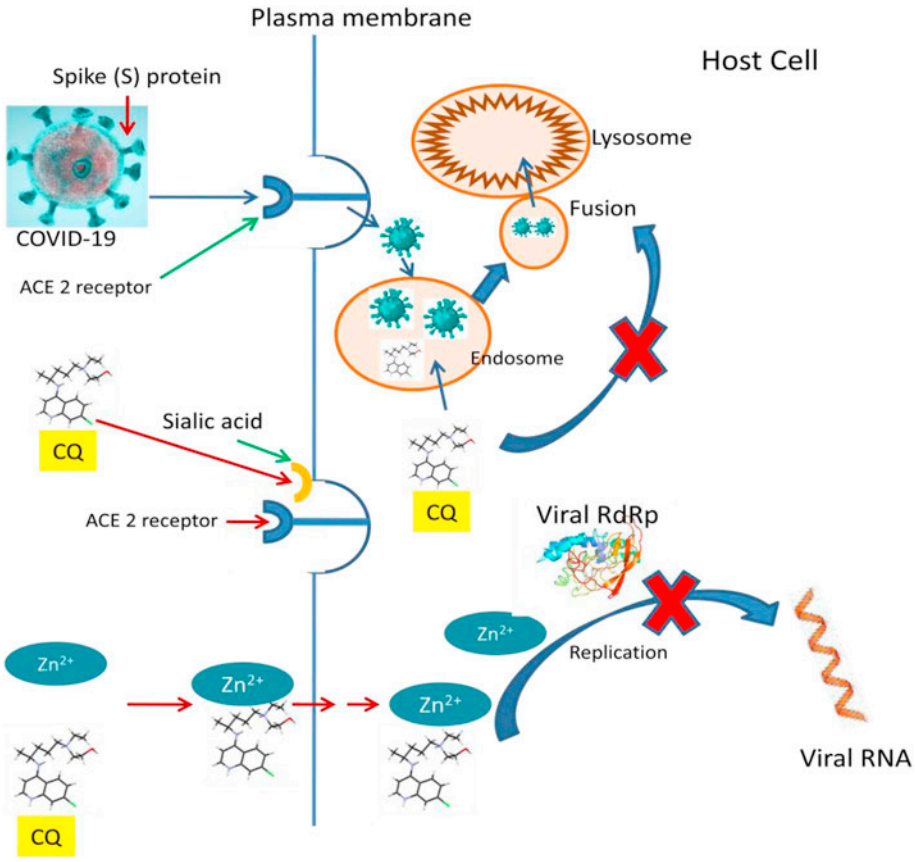


Fig. 4. Proposed mechanism of action for aminoquinolines (CQ: Chloroquine; RdRp: RNA dependent RNA polymerase; Green color arrow: names; Red color arrow: Zn2+ ionophore action of CQ; Blue color arrow: COVID-19 entry into host cell, endosome and lysosome; X: Site of action for CQ). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

binding with Sialic acid [112–114]. The three mechanisms are illustrated in Fig. 4.

3. Chloroquine vs. hydroxychloroquine

When compared for pharmacological profile, chloroquine and hydroxychloroquine possess equivalent anti-malarial activity. The latter is preferred due to lower ocular toxicity [115]. Theoretically, the antiviral activity for chloroqunie and hydroxychloroquine are similar but the reported clinical details for chloroquine are more in number [116]. Use of chloroquine is less due to some associated adverse effects and lack of availability in some countries. In patients with COVID-19 infection hydroxychloroquine is preferred as chloroquine when used in combination with lopinavir or ritonavir shows prolongation of the QT interval. Some of the other antiviral therapeutics that does not interfere with hydroxychloroquine is oseltamivir, lopinavir/ritonavir, robavirin, interferons, and immunoglobulins (intravenous) [117].

4. Safety and adverse effects

The toxicological properties reported with use of chloroquine and hydroxychloroquine are retinopathy, neuromyopathy and cardiomyopathy. Both these drugs possess affinity for melanin and affect the macular cones. The phagocytic activity of lysosomes is declined on the photoreceptors and they migrate towards central and peripheral regions as well as induces epithelial atrophy and irreversible alterations in photoreceptors [106]. In the lysosomes, hydroxychloroquine is protonated and accumulated due to its basic nature. It inhibits the activity of lysosomal phospholipases causing vacuolization of cardiac and skeletal muscle cells [118]. Prolong use of hydroxychloroquine produces – i) toxicity to retina tissue which may lead to unrepirable retinopathy [119,120]; ii) Cardiotoxicity and CNS toxicity with neuromyopathy symptoms and alterations in gastrointestinal tract [121]; toxicity to liver cells (genetic material) [122]; and genotoxicity [123,124]. Studies have shown substantial increase in retinal toxicity with chronic treatment based on the hypothesis of bioaccumulation [125]. Some investigators report $<2\%$ incidences of retinopathy and more common in Asian patients [126,127]. Some studies failed to report cardiac

complications and neuromyopathy and may be rare after long-term treatment [128,129]. The monitoring of side effects need to be continued even after discontinuation of treatment due to prolong half-life of chloroquine and hydroxychloroquine. The side effects (keratopathy, maculopathy) may be delayed. The current anti-COVID-19 therapeutic regimen suggests longer duration of treatment with chloroquine than that as anti-malarial drug. Thus close monitoring of the adverse reactions, pharmacological effects, poisoning and toxicological mechanisms to provide help to the worldwide clinical work [110].

Consideration of clinical outcomes is essential to be monitored to design safe and effective protocol with prevention of toxicological effects for therapeutic use of the antiviral aminoquinolines (chloroquine and hydroxychloroquine).

5. Outcomes of in vivo, in vitro studies and clinical trials

WHO has framed a collective protocol to conduct randomized clinical trials for investigating the clinical role and safety of therapeutics for the treatment of COVID-19 infection [130]. As on 14 April 2020, approximately 961 clinical trials which are carrying on worldwide have been reported to WHO. The essential ethical requirement is use of chloroquine in COVID-19 patients with ethical trial approval or off-label. Timely availability of the clinical outputs to the biomedical fraternity is important considering the evolving outbreak and growing number of COVID-19 infected patients with availability of any specific licensed drug. The use of chloroquine in the treatment of COVID-19 infection is considered by WHO as experimental. In this regard use of chloroquine is associated with various concerns such as patient safety, close monitoring of drug use, etc. The repurposing of the anti-malarial drugs need to follow ethical approaches and may raise concern about shortage of such drugs. The outcomes of some of the reported in vivo, in vitro and clinical studies carried globally have been documented here one by one. Approximately 100 clinical trials are in recruiting or pending approval and are ongoing at single or multiples centers with satisfactory primary outcomes but final outcomes/results are pending. These are summarized in Table 1 for ease of access to medical fraternity.

- In vitro study of chloroquine were performed in Vero E6 cells infected with SARS-CoV with a multiplicity of infection (0.05) demonstrated effective reduction of viral replication (EC_{90} = 6.90 μM). The antiviral effects are reproducible with standard dose with favorable tissue and lung penetration. The proposed mechanism of viral inhibition involves increased pH in endosomes, altered glycosylation of SARS-CoV cellular receptor and the synergistic action of immunomodulation properties [131].
- The Department of Science and Technology (Guangdong Province and Health Commission of Guangdong Province) reported a multicentric collaborative in vitro and clinical study, use of chloroquine phosphate (dose 500 mg twice a day for 10 days) in mild, moderate and severe SARS-CoV-2 pneumonia [132]. The study included some advisories to monitor for history of drug contraindication, blood testing for anemia, thrombocytopenia or leucopenia, serum electrolyte disturbances, tests for hepatic and renal functioning, routine electrocardiography and monitoring for visual and mental disturbances. Concurrent administration of some drugs should avoided including drugs which can prolong QT interval (examples: quinolones, macrolides, ondansetron), anti-arrhythmic, anti-depressant and antipsychotic drugs.
- The Italian Society of Infectious and Tropical disease, Lombardy section suggests administration of chloroquine (500 mg, twice a day) or hydroxychloroquine (200 mg per day for 10 days) (5 to 20 days treatment depending on clinical severity) [133].
- Another guideline as documented by the Dutch Center of Disease Control (CDC) recommended use of chloroquine to treat severe infections with requirement of oxygen therapy and optimal supportive care [134]. The recommended dose for chloroquine base is 600 mg followed by 300 mg after 12 h (on day 1) and 300 mg twice a day (for 2–5 days) and discontinuation of the treatment at day 5 to reduce the side effects (30 h half-life of chloroquine) (500 mg of chloroquine phosphate = 300 mg of chloroquine base).
- In China, more than two dozen clinical trials have been carried out for evaluating efficacy various anti-viral drugs in different disease severity to investigate dose and duration of treatment. The studies have been coordinated by the Chinese authorities through a prescribed regulating guideline [135]. These trials are the first to report the characteristics and management of COVID-19 infected patients but no details on use of chloroquine [136–140].

6. Conclusion

For therapeutic use of aminoquinolines (chloroquine and hydroxychoroquine) the important aspects are – i) it will be administered to millions of infected patients with COVID-19, ii) It will be administered to medical workers as preventive measure, iii) during acute approach against COVID-19 higher dose will be administered as compared to use during the treatment of chronic rheumatic diseases [141]. Following points can be concluded and to be considered during the use of aminoquinolines (chloroquine and hydroxychloroquine) for the treatment of COVID-19 infection –

i) History of previous or ongoing use of chloroquine and hydroxychloroquine in malaria, amebiasis, rheumatoid arthritis, and systemic lupus;
ii) Higher risk of development of retinopathy in Asian patients;
iii) Periodical monitoring of patients with vision problems, cardiovascular problems;
iv) To measure central and peripheral visual acuity;
v) Drug interaction with Kaolin clay and antacids reduces antiviral and anti-inflammatory action;
vi) Regular monitoring for symptoms like ocular pruritus and cardiac arrhythmias;
vii) Aminoquinolines decreases activity of immunosupressants and antibiotics;
viii) Other aminoquinoline analog, Mefloquine is associated with increased risk of convulsion;
ix) The toxicity is associated with the dose calculated by real weight and therefore dose should be suitable for patients with potential high risk of adverse effects. Cumulative dose >203 mg/kg body weight/day is under high risk category. [142].

In the absence of sufficient clinical data, detailed information on safety, adverse effects, dose of aminoquinolines (chloroquine and hydroxychloroquine), etc. should be made available among health professionals who will dissipate it among patients. The successful application of available resources needs to be grounded in practices to minimize risk of rigorous screening and dose calculation.

Acknowledgment

Authors acknowledge the resources provided by KIET Group of Institutions (Delhi-NCR), Lala Lajpat Rai Memorial Medical College (Meerut) and Dr. A. P. J. Abdul Kalam Technical University (Lucknow), India for the completion of work.

Funding

The work is not supported by any funding agency.

Declaration of competing interest

Authors declare no potential conflict of interest.

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FOI 26-3132 - Document 5

Europ.J.clin.Pharmacol. 9, 131-134 (1975)
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Effect of Zinc Sulphate on the Absorption of Tetracycline and Doxycycline in Man

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Received: April 23, accepted: May 31, 1975

Summary. A possible interaction between zinc sulphate and tetracycline or doxycycline was examined in a cross-over study in seven volunteers. A single dose of zinc sulphate (45 mg Zn^{++}) was given simultaneously with tetracycline hydrochloride (500 mg) or doxycycline chloride (200 mg). The serum concentration of tetracycline, the area under the serum tetracycline concentration-time curve and the excretion of tetracycline in urine were reduced by about 30% ($p < 0.05$) from the respective control values. The absorption of doxycycline was not influenced significantly by zinc sulphate. The clinical significance of the zinc-tetracycline interaction seems to be of limited importance.

Key words: Drug interaction, drug absorption, zinc sulphate, tetracycline, doxycycline.

It is well documented that the gastrointestinal absorption of various tetracycline derivatives is affected by many di- and trivalent cations, e.g. simultaneous ingestion of aluminium, magnesium, calcium or iron may interfere with the absorption of tetracyclines (8, 10, 12). Similarly, absorption of the various cations may also be inhibited by the interaction (4, 9), which is based on formation of tetracycline-metal complexes in the gut. The ability of various tetracycline derivatives to form chelates *in vitro*, e.g. with calcium, varies considerably (11). Furthermore, the absorption of doxycycline is not significantly affected by milk in conditions when the absorption of tetracycline is markedly inhibited (6). On the contrary, the simultaneous intake of iron causes much more reduction of the absorption of doxycycline than of tetracycline (9).

Oral zinc sulphate has been introduced recently into the treatment of leg ulcers (3), acrodermatitis (7) and to hasten wound healing in situations of increased zinc loss or reduced intake (2). Because zinc sulphate and tetracycline derivatives may be used simultaneously in certain clinical situations and since zinc is able to form chelates with tetracyclines *in vitro* (1), a study has been made of the effect of therapeutic doses of zinc on the absorption of tetracycline and doxycycline.

PATIENTS AND METHODS

A cross-over study was performed in four healthy persons and in three long-stay patients who volunteered for the experiment. The ages of the subjects ranged from 29 to 85 years, and their weights from 44 to 75 kg. Two of the patients were on digoxin 0.25 mg daily. All the volunteers received the usual hospital diet during the study. The

FOI 26-3132 - Document 5

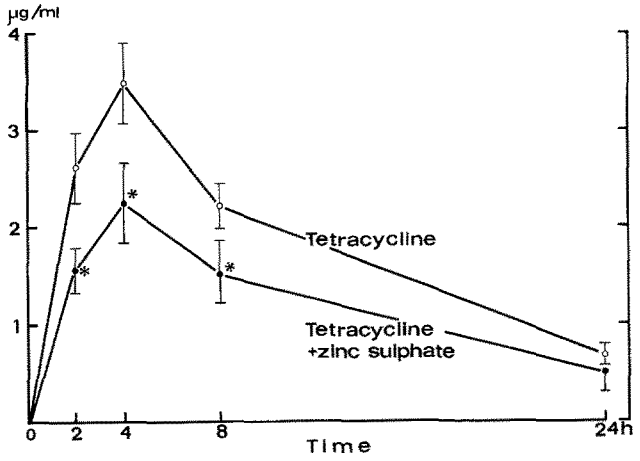


Fig. 1. Serum concentration of tetracycline after ingestion of tetracycline hydrochloride (500 mg) alone or simultaneously with zinc sulphate (200 mg, containing 45 mg Zn^{++}). Means (± S.E.) in a cross-over study in seven persons are shown. The asterisk indicates a significant difference ($p<0.05$) between the appropriate results

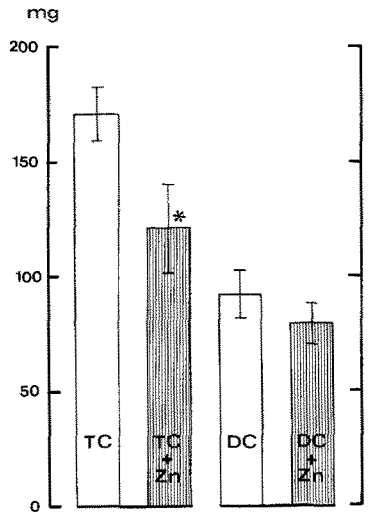


Fig. 2. Effect of simultaneous ingestion of zinc sulphate (containing 45 mg Zn^{++}) and tetracycline hydrochloride (500 mg) or doxycycline chloride (200 mg as a base) on 48 hour excretion of the antibiotics in urine (Mean ± S.E.; n = 7). The asterisk indicates a significant difference ($p<0.05$) between the appropriate results

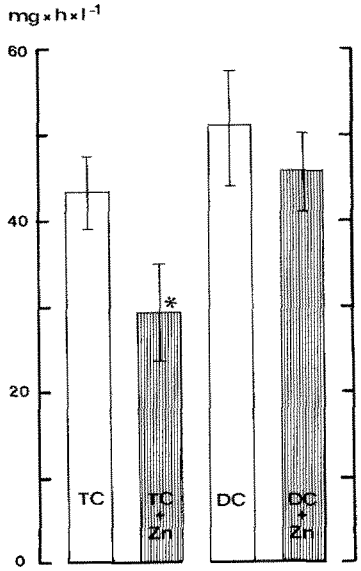


Fig. 3. Effect of simultaneous ingestion of zinc sulphate (containing 45 mg Zn^{++}) and tetracycline hydrochloride (500 mg) or doxycycline chloride (200 mg as a base) on the areas under individual serum antibiotic concentration-time curves (Mean ± S.E.; n = 7). The asterisk indicates a significant difference ($p<0.05$) between the appropriate areas

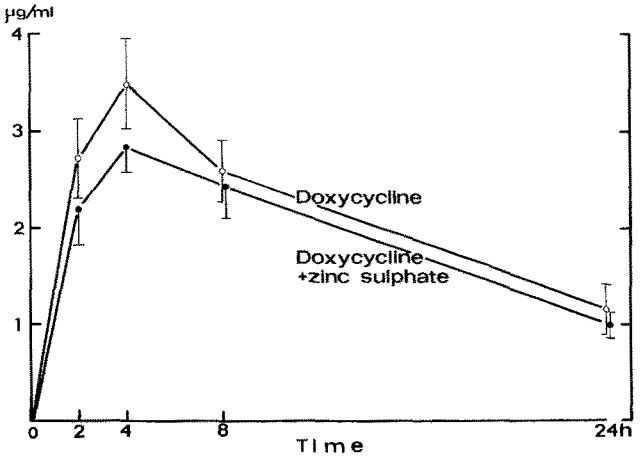


Fig. 4. Serum concentration of doxycycline after ingestion of doxycycline chloride (200 mg as a base) alone or simultaneously with zinc sulphate (containing 45 mg Zn^{++}). Means (± S.E.) in a cross-over study in seven persons are shown

FOI 26-3132 - Document 5

drugs were administered after an overnight fast. A light meal without any dairy products was served three hours after taking the drugs.

The experiment was carried out according to the following plan: according to a cross-over design the volunteers took in random order a single dose of two capsules of tetracycline hydrochloride 250 mg (caps. Medicyclin, Medica Ltd., Helsinki) simultaneously with either 200 ml water or 200 mg zinc sulphate (containing 45 mg Zn^{++}; Solvezink, Draco Ltd., Lund) in 200 ml of water. The interval between the two experiments was one week. After a further week the second cross-over study which was of identical design, was started using the other tetracycline derivative, doxycycline chloride (caps. Vibramycin, Pfizer Corp., Brussels).

Blood samples were taken 0, 2, 4, 8 and 24 h after ingestion of the drugs Urine was collected in 24 hour fractions for 2 days. The pH of the fresh morning urine was determined. Serum and urine samples were stored at -18°C until analysed by a fluorometric method (5). The relative areas under the individual serum tetracycline and doxycycline concentration-time curves during 24 h were estimated.

The means and standard errors (S.E.) were calculated and the results were analysed statistically by Student's t-test for paired values.

RESULTS

Simultaneous ingestion of zinc sulphate and tetracycline resulted in a 30-40 per cent reduction in the serum concentration of tetracycline, the difference from the control group being significant ($p < 0.05$) at 2, 4 and 8 hours after treatment (Fig. 1). The area under the serum tetracycline concentration-time curve and the 48 hour excretion of tetracycline in urine were significantly less ($p < 0.05$) when tetracycline was taken with zinc than without it (Figs. 2 and 3).

The absorption of doxycycline was not significantly affected by simultaneous ingestion of zinc sulphate 200 mg, as judged by the serum doxycycline concentration, by the area under the serum doxycycline concentration-time curve and by the excretion of doxycycline in urine (Figs. 2, 3 and 4).

The pH of fresh morning urine was not significantly different between various groups.

DISCUSSION

The present results demonstrate that absorption of tetracycline was inhibited to some degree whereas the absorption of doxycycline was not affected significantly by simultaneous treatment with zinc sulphate.

Recently oral zinc sulphate has been introduced into the therapy of certain surgical and dermatological situations including, for example, promotion of wound healing after operations and burns, venous leg ulcers and acrodermatitis enteropathica as well as certain other disorders of cutaneous healing in patients with zinc deficiency (2, 3, 7). In these conditions the use of tetracycline derivatives may sometimes be indicated and their absorption is likely to be inhibited if both drugs are taken together. It is reasonable to assume that they interact by formation of zinc-tetracycline complexes in the gut, as demonstrated *in vitro* by Albert (1). The absorption of zinc would also be decreased as a result of the mutual interaction, as in the reported iron-tetracycline interaction (4, 9).

It has been shown previously that the interaction between tetracycline and iron can be avoided by leaving a certain time interval between the doses (6). Zinc and tetracycline seem unlikely to affect each other if there is a 2-3 hour interval between the doses.

The affinity with which various tetracycline derivatives form chelates *in vitro* with different cations varies considerably (11). The absorption of doxycycline, a derivative with the lowest affinity for calcium is affected only to a small extent, or not at all (6), by milk in conditions where the absorption of tetracycline is significantly inhibited. On the other hand, simultaneous intake of iron causes much greater reduction of the absorption of doxycycline than of tetracycline (8). Thus, zinc resembles calcium more closely than iron in that it has very little effect on the absorption of doxycycline.

In conclusion, there is a statistically significant interaction between zinc sulphate and tetracycline when they are ingested simultaneously. However, the reduction of serum tetracycline or doxycycline concentration by zinc sulphate is so small that their interaction can only be of limited clinical significance.

Acknowledgements. The authors are indebted to the volunteers and the nurses for their essential assistance.

FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Zinc in Human Health: Effect of Zinc on Immune Cells

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Although the essentiality of zinc for plants and animals has been known for many decades, the essentiality of zinc for humans was recognized only 40 years ago in the Middle East. The zinc-deficient patients had severe immune dysfunctions, inasmuch as they died of intercurrent infections by the time they were 25 years of age. In our studies in an experimental human model of zinc deficiency, we documented decreased serum testosterone level, oligospermia, severe immune dysfunctions mainly affecting T helper cells, hyperammonemia, neurosensory disorders, and decreased lean body mass. It appears that zinc deficiency is prevalent in the developing world and as many as two billion subjects may be growth retarded due to zinc deficiency. Besides growth retardation and immune dysfunctions, cognitive impairment due to zinc deficiency also has been reported recently. Our studies in the cell culture models showed that the activation of many zinc-dependent enzymes and transcription factors were adversely affected due to zinc deficiency. In HUT-78 (T helper 0 (Th_0) cell line), we showed that a decrease in gene expression of interleukin-2 (IL-2) and IL-2 receptor α (IL-2Rα) were due to decreased activation of nuclear factor-κB (NF-κB) in zinc deficient cells. Decreased NF-κB activation in HUT-78 due to zinc deficiency was due to decreased binding of NF-κB to DNA, decreased level of NF-κB p105 (the precursor of NF-κB p50) mRNA, decreased κB inhibitory protein (IκB) phosphorylation, and decreased Iκκ. These effects of zinc were cell specific. Zinc also is an antioxidant and has anti-inflammatory actions. The therapeutic roles of zinc in acute infantile diarrhea, acrodermatitis enteropathica, prevention of blindness in patients with age-related macular degeneration, and treatment of common cold with zinc have been reported. In HL-60 cells (promyelocytic leukemia cell line), zinc enhances the upregulation of A20 mRNA, which, via TRAF pathway, decreases NF-κB activation, leading to decreased gene expression and generation of tumor necrosis factor-α (TNF-α), IL-1β, and IL-8. We have reported recently that in both young adults and elderly subjects, zinc supplementation decreased oxidative stress markers and generation of inflammatory cytokines.

Online address: http://www.molmed.org
doi: 10.2119/2008-00033.Prasad

INTRODUCTION

In 1869, Raulin (1) first showed that zinc was required for the growth of Aspergillus niger. In 1934, Todd *et al*. (2) reported that zinc was essential for the growth of rats. Although the essentiality of zinc for animals was established, its ubiquity made it seem improbable that zinc deficiency in humans could lead to any significant clinical problem. During the past 40 years, however, it has become apparent that deficiency of zinc in humans is quite prevalent and may affect over two billion subjects in the developing world (3–5). Here, we review some of the clinical manifestations of zinc deficiency in humans, with particular emphasis on our observations in experimental human models of this syndrome.

CLINICAL MANIFESTATIONS OF ZINC DEFICIENCY

During the past four decades, a spectrum of clinical deficiency of zinc in human subjects has emerged (6). On the one hand, the manifestations of zinc deficiency may be severe; and, on the other end of the spectrum, zinc deficiency may be mild or marginal. A severe deficiency of zinc has been reported to occur in patients with acrodermatitis enteropathica (a genetic disorder), following total parenteral nutrition (TPN) without zinc, following excessive use of alcohol, and following penicillamine therapy. The manifestations of severe zinc deficiency in humans include bullous pustular dermatitis, alopecia, diarrhea, emotional disorder, weight loss, intercurrent infections due to cell-mediated immune dysfunctions, hypogonadism in males, neurosensory disorders, and problems with healing of ulcers. If this condition is unrecognized and untreated, it becomes fatal.

The manifestations of a moderate deficiency of zinc include growth retardation and male hypogonadism in adolescents, rough skin, poor appetite, mental lethargy, delayed wound healing, cell-mediated immune dysfunctions, and abnormal neurosensory changes.

In our studies in the experimental human model in whom only a mild deficiency of zinc in males was induced by dietary means, decreased serum testosterone level, oligospermia, decreased natural killer (NK) cell activity, decreased interleukin-2 (IL-2) production, decreased thymulin activity, hyperammonemia, hypogeusia, de-

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Submitted March 17, 2008; Accepted for publication March 24, 2008; Epub (www.molmed.org) ahead of print April 3, 2008.

FOI 26-3132 - Document 5

creased dark adaptation, and decreased lean body mass were observed. It is, therefore, clear that even a mild deficiency of zinc in humans affects clinical, biochemical, and immunological functions adversely (7–9).

Nutritional deficiency of zinc in the developing countries is caused by ingestion of high cereal protein intake, rich in phytate (an organic phosphate compound), which makes zinc unavailable for absorption. Other causes of zinc deficiency include malabsorption syndrome, hyperzincuria as seen in cirrhosis of the liver and sickle cell disease, blood loss due to hookworm infection, and excessive sweating in hot tropical climates.

In 1974, a landmark decision to establish recommended dietary allowances (RDAs) for humans for zinc (15 mg elemental zinc daily for adult men and women) was made by the Food and Nutrition Board of the National Research Council of the United States of America National Academy of Sciences.

ZINC AND IMMUNITY

Zinc affects multiple aspects of the immune system (10). Zinc is crucial for normal development and function of cells mediating innate immunity, neutrophils, and NK cells. Macrophages also are affected by zinc deficiency. Phagocytosis, intracellular killing, and cytokine production all are affected by zinc deficiency. Zinc deficiency adversely affects the growth and function of T and B cells. The ability of zinc to function as an antioxidant and stabilize membranes suggests that it has a role in the prevention of free radical-induced injury during inflammatory processes.

STUDIES OF IMMUNE FUNCTIONS IN EXPERIMENTAL HUMAN MODEL

During our studies in the Middle East, we observed that most of the zinc deficient dwarfs did not live beyond the age of 25 years. The cause of death appeared to be infections. Parasitic infections were common. Viral and bacterial infections, although prevalent, remained undocumented, however. The possibility that zinc deficiency may have played a role in immune dysfunctions in the zinc-deficient dwarfs was considered, but lack of proper facilities prevented us from gathering meaningful data on immune functions in those patients in the Middle East.

In our experimental human model studies, when zinc deficiency was very mild (3 to 5.0 mg Zn intake during the zinc-restricted period), the plasma zinc concentration remained more or less within the normal range and it decreased only after 4–5 months of zinc restriction. On the other hand, zinc concentrations in lymphocytes, granulocytes, and platelets decreased within 8–12 weeks, suggesting that the assay of cellular zinc provided a more sensitive criterion for diagnosing mild deficiency of zinc (7–9).

We assayed serum thymulin activity in mildly zinc-deficient human subjects (8). Thymulin is a thymus-specific hormone and it requires the presence of zinc for its biological activity to be expressed. Thymulin binds to high-affinity receptors on T cells, induces several T-cell markers, and promotes T-cell function, including allogenic cytotoxicity, suppressor functions, and interleukin-2 (IL-2) production. As a result of mild deficiency of zinc, the activity of thymulin in serum was decreased significantly and was corrected by both *in vivo* and *in vitro* zinc supplementation (7). The *in vitro* supplementation studies indicated that the inactive thymulin peptide was present in the serum in zinc-deficient subjects and was activated by the addition of zinc.

For the quantification of T cells, beads coated with T_{101} Mab and, for B lymphocytes, beads coated with anti human immunoglobulin were used, (Quantigen, Bio-Rad laboratories, Richmond, CA, USA) 1×10^6 mononuclear cells (MNC) were incubated with 200 μL of one or two microbeads preparation. Cells with no beads were counted as T_{101}–, slg–. An increase in T_{101}–, slg- cells, a decrease in the ratio of $CD4^+$ to $CD8^+$, and decreased IL-2 activity (proliferation assay [7]) were observed in the experimental human model during the zinc-depletion phase, all of which were corrected after repletion with zinc. We previously had reported that NK cells' activity was also sensitive to zinc restriction, thus it appears that zinc may play a very important and critical role in the functions of T cells in humans (6,10). Our studies in the experimental human model showed for the first time that the production of IFN-γ was decreased, whereas the production of IL-4, IL-6 and IL-10 was not affected due to zinc deficiency (8,9). IFN-γ is known to downregulate the T helper 2 (Th_2) clone, and IL-10 may downregulate the T helper 1 (Th_1) clone. An imbalance between Th_1 and Th_2 responses in patients with human immunodeficiency virus infection has been implicated in the immune dysregulation in these patients, and it has been proposed that resistance to infection and/or progression to acquired immunodeficiency syndrome is dependent on a $Th_1 > Th_2$ dominance. Our data in experimental human models suggest that cell-mediated immune dysfunctions in human zinc deficiency may be due to an imbalance between Th_1 and Th_2 cell functions.

Th_1 cells are known to promote macrophage activation and production of complement fixing and opsonizing antibodies. IFN-γ is the major component of the Th_1 response panel, and it upregulates major histocompatibility complex class I antigen expression. Inasmuch as we have observed that IFN-γ production is decreased as a result of zinc deficiency, our studies provide a possible mechanism by which zinc deficiency may affect cell-mediated immunity adversely.

Several studies have shown the benefits of zinc supplementation on infectious diseases in humans. In double-blind placebo-controlled trials of zinc supplementation, zinc reduced the incidence and duration of acute and chronic diarrhea and acute lower respiratory tract infections in infants and children (11,12). Zinc supplementation of sickle cell anemia patients in a placebo-controlled trial resulted in decreased incidence of staphylococcus aureus pneumonia, streptococcus pneumonia tonsillitis, and *E. coli* urinary tract infections (13). Our recent studies

FOI 26-3132 - Document 5

have shown that zinc supplementation to elderly subjects results in a significant decrease in the incidence of infections (14).

ZINC ACTIVATES NF-κB IN HUT-78 CELLS

The current estimate is that over 2,000 transcription factors may be zinc dependent. However, the present dogma is that the amount of zinc required for the integrity of these transcription factors is so small that, in zinc-deficient cells, one would not see any change.

Because zinc deficiency affects IL-2 production and T-cell activation adversely, we have investigated the role of zinc on NF-κB (nuclear factor-κB) activation in HUT-78, a T helper 0 (Th_0) human malignant lymphoblastoid cell line. We showed for the first time that, in zinc deficient HUT-78 cells, the activation of NF-κB was affected adversely (15).

PHOSPHORYLATION AND UBIQUITINATION OF IκB

Intracellular zinc concentration was decreased by 40% in zinc-deficient HUT-78 cells in comparison to the zinc-sufficient cells after 4 d of incubation. To examine the effect of zinc on κB inhibitory protein (IκB) in HUT-78 cells, we measured phosphorylated IκB-α, Iκκ-α (IκB kinase-α), and ubiquitinated IκB-α in zinc-treated cells. Zinc deficiency caused a 60% decrease in phosphorylated IκB-α in non-stimulated and 40% decrease in phorbol-12 myristate 13 acetate (PMA)/phytohemagglutinin-p (PHA-p)–stimulated cells, compared with zinc-sufficient cells. Zinc deficiency decreased Iκκ-α in PMA/PHA-p–stimulated cells by 30% in comparison to zinc-sufficient cells. Zinc deficiency caused a 50% reduction of ubiquitinated IκB-α after PMA/PHA-p stimulation (15).

We showed that the phosphorylation of IκB and Iκκ, translocation of NF-κB, and its binding to DNA in HUT-78 cells were all zinc dependent (15). The activation of NF-κB was considerably reduced in zinc-deficient HUT-78 cells. Additionally, zinc deficiency decreased the level of NF-κB p105 (the precursor of NF-κB p50) mRNA. All these effects of zinc were cell specific.

ZINC ENHANCES THE EXPRESSION OF IL-2 AND IL-2 RECEPTORS IN HUT-78 CELLS VIA NF-κB ACTIVATION

The production of interleukin-2 (IL-2) is a key and early event in the activation of T-lymphocytes. IL-2 triggers peripheral T-lymphocytes to enter the S phase of the cell cycle and to divide. This is probably a result of the suppressive effect of IL-2 on cell cycle inhibitors, which interfere with the activity of cyclin-dependent kinases (cdks) at checkpoints of the cell cycle (16). IL-2 also is involved in the differentiation of thymocytes, peripheral T- and B-lymphocytes and other cells of hematopoietic origin (16).

A short segment of DNA, 275 base pairs in the promoter area of IL-2 gene, integrates numerous signaling pathways leading to IL-2 synthesis and the activation and proliferation of T-lymphocytes (16). Both the murine and human IL-2 promoters contain one binding site for genuine Rel/NF-κB factors. The sequence of this site, GGGATTTCAC, is identical for both promoters. NF-κB factors are induced rapidly by a variety of stimuli activating T cells. Almost every stimulus leading to T cell activation also activates NF-κB. Induction of IL-2Rα gene expression also is mediated by the induced nuclear expression of NF-κB (17,18).

IL-2 AND SOLUBLE IL-2Rα PRODUCTION

The details of cell culture, zinc assay, and other methods have been published elsewhere (19). We used an Enzyme-Linked ImmunoSorbent Assay (ELISA) to measure the concentration of IL-2 produced by the cells. Unstimulated HUT-78 cells incubated in zinc-deficient (1 μmol/L zinc) and zinc-sufficient (15 μmol/L zinc) media for 4 d produced a very small amount of IL-2. Following PMA/PHA-p stimulation, the IL-2 production and IL-2 mRNA were significantly greater in zinc-sufficient cells in comparison to the zinc-deficient cells (19). Similar results were seen for soluble IL-2 receptor α (soluble IL-2Rα) production and gene expression (19).

ZINC DECREASES OXIDATIVE STRESS AND INFLAMMATORY CYTOKINES

The role of zinc in modulating oxidative stress has recently been recognized. Oxidative stress is an important contributing factor in several chronic human diseases, such as atherosclerosis and related vascular diseases, mutagenesis and cancer, neurodegeneration, immunologic disorders, and the aging process (20–22). Together $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$ are known as reactive oxygen species (ROS), and these are produced continuously *in vivo* under aerobic conditions. The NADPH oxidases are a group of plasma membrane associated enzymes, which catalyze the production of $O_2^{\cdot-}$ from oxygen by using NADPH as the electron donor. Zinc is an inhibitor of this enzyme. The dismutation of $O_2^{\cdot-}$ to H_2O_2 is catalyzed by an enzyme super oxide dismutase (SOD), which contains both copper and zinc. Zinc is known to induce the production of metallothionein, which is very rich in cysteine, and is an excellent scavenger of $^{\cdot}OH$ (6). Iron and copper ions catalyze the production of $^{\cdot}OH$ from H_2O_2. Zinc is known to compete with both iron and copper for binding to cell membrane, thus decreasing the production of $^{\cdot}OH$ (6).

A few investigators have reported that inflammatory cytokines such as TNF-α (tumor necrosis factor-α) and IL-1β, generated by activated monocytes-macrophages, also are known to produce increased amounts of ROS (23,24). Increases in these cytokines are associated with decreased zinc status in patients.

NF-κB is involved in the expression of a variety of responsive specific genes and is activated by several stimuli such as cytokines, radiation, and oxidative stress. *In vitro* activation of NF-κB by TNF-α in MNC has been shown to be an excellent model of oxidative stress-sensitive transactivating factor, and has been used to evaluate the efficacy of compounds in protecting cells from oxidative stress (21). Zinc has been shown to inhibit NF-κB activation in prostate cancer cells, thus enhancing anti-cancer therapy (25), bovine cerebral epithelial

FOI 26-3132 - Document 5

cells (26), as well as reducing increased levels of activated NF-κB in diabetic CD1 mice (27) and zinc deficient cultured human hepatocellular carcinoma-derived cell line (28).

The induction of NF-κB activation pathway appears to be cell specific and is counterbalanced by concomitant activation of NF-κB activation inhibitors. One such inhibitor of NF-κB activation is A20, a zinc finger-transactivating factor which also binds to DNA, producing the A20 protein which inhibits TNF-α-induced NF-κB activation (29–33). A20 plays an important role in reducing IL-1β- and TNF-α-induced NF-κB activation (29–33).

Our data showed that zinc supplementation to normal healthy subjects a) lowers the oxidative stress-related by-products MDA (malondialdehyde), 4-hydroxyalkenals (HAE), and 8-hydroxy deoxyguanine (8-OHdG) generated by cells and released into the plasma, b) inhibits the induction of TNF-α and IL-1β mRNA in MNCs, and c) exhibits a protective effect against TNF-α-induced NF-κB activation in isolated MNCs (34). In addition, we provided evidence to show that, in the human pro-myelocytic leukemia cell line HL-60 which differentiates to the monocyte-macrophage phenotype by PMA, zinc increased the expression of A20 and the binding of A20 transactivating factor to DNA, thereby enhancing inhibition of induced NF-κB activation (34).

The role of zinc in regulation of the gene expression of IL-1β and TNF-α has not been defined. The zinc finger protein A20 has been shown to inhibit NF-κB signaling by TNF-α and IL-1β via TNF-receptor gene (TRAF pathways) in endothelial cells (31,35). A20 is expressed in various types of cells in response to a number of stimuli such as TNF-α, IL-1β, LPS (lipopolysaccharide), PMA, Epstein-Barr virus latent membrane protein, as well as other stimuli (32). A20 expression primarily protects cells from TNF-α-induced cytotoxicity by decreasing the activation of NF-κB, which leads to decreased IL-1β and TNF-α gene expression as has been demonstrated in endothelial cells. We propose that a similar effect of zinc supplementation on the A20 pathway occurs in primary cells. Further studies are ongoing in our laboratory to aid in the understanding the mechanisms of zinc action.

Our study provides molecular evidence for an anti-oxidant effect of zinc in human subjects and shows that zinc supplementation *in vivo* protected MNC against oxidative stress (34). Although there are several possible biochemical mechanisms by which zinc may decrease oxidative stress in cells, our study shows that zinc negatively regulates gene expression of inflammatory cytokines such as TNF-α and IL-1β, which are known to generate ROS and this may be one additional mechanism by which zinc may be functioning as an antioxidant in humans. Thus, our study provides rationale for use of zinc in therapeutic trials either alone or in conjunction with other modalities in chronic diseases, including chemo-prevention of cancer in which oxidative stress is known to play an important role.

The immunological hallmarks of zinc deficiency in humans and higher animals include thymic atrophy, lymphopenia, and compromised cell- and antibody-mediated responses that result in increased incidences of infections (36). In chronic zinc deficiency, a reprogramming of the immune system occurs, beginning with the activation of the stress axis and chronic production of glucocorticoids that accelerate apoptosis of pre-B and pre-T cells (36), resulting in reduced lymphopoeisis and atrophy of thymus. In contrast, myelopoesis is preserved. Changes in the gene expression for cytokines, DNA repair enzymes, zinc transporters, and signaling molecules, suggest that the cells of the immune system are attempting to adapt to the stress of suboptimal zinc (36).

We have observed that, as a result of zinc deficiency, the macrophages-monocytes are stressed and they generate inflammatory cytokines such as TNF-α and IL-1β (14,34). In a recent study, we reported that, in comparison to the younger adults, the elderly subjects had lower plasma zinc, increased oxidative markers, and increased generation of inflammatory cytokines (14). Following zinc supplementation to the elderly subjects, the plasma zinc increased, oxidative stress markers decreased, and generation of inflammatory cytokine decreased, in comparison to the placebo group.

In a large study organized by the National Eye Institute, NIH, (Bethesda, MD, USA) it was reported that zinc and antioxidants (vitamin C, vitamin E, and β carotene) significantly reduced the odds of developing advanced age related macular degeneration (AMD), and prevented blindness in the high-risk group of elderly subjects (37). Also, it was reported that longevity was increased in the zinc supplemented group (38). Although the mechanism of zinc effect was not defined, one may hypothesize that zinc reduced the oxidative stress and thus was beneficial in AMD.

ROLE OF ZINC IN SUPPRESSING ALLOGENEIC REACTION WITHOUT AFFECTING THE ANTIGENIC RESPONSE

Faber *et al*.(39) have shown that *in vivo* zinc supplementation in therapeutic levels resulted in blocking allogeneic response while maintaining the antigenic potency. This observation may have future implication in transplantation subjects where a selective suppression of allogeneic response is desired.

ACKNOWLEDGMENTS

Grant support for this work was provided in part by NIH grant 5 RO1 A150698-04 and Labcatal Laboratories, Paris, France.

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FOI 26-3132 - Document 5

Communication

DOI: 10.5582/ddt.2021.01005

Multidrug treatment for COVID-19

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SUMMARY An outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which began in Wuhan, China in December 2019, has rapidly spread all over the world. The World Health Organization characterized the disease caused by SARS-CoV-2 (COVID-19) as a pandemic in March 2020. In the absence of specific treatments for the virus, treatment options are being examined. Drug repurposing is a process of identifying new therapeutic uses for approved drugs. It is an effective strategy to discover drug molecules with new therapeutic indications. This strategy is time-saving, low-cost, and has a minimal risk of failure. Several existing approved drugs such as chloroquine, hydroxychloroquine, doxycycline, azithromycin, and ivermectin are currently in use because of their efficacy in inhibiting COVID-19. Multidrug therapy, such as a combination of hydroxychloroquine and azithromycin, a combination of doxycycline and ivermectin, or a combination of ivermectin, doxycycline, and azithromycin, has been successfully administered. Multidrug therapy is efficacious because the mechanisms of action of these drugs differ. Moreover, multidrug therapy may prevent the emergence of drug-resistant SARS-CoV-2.

Keywords hydroxychloroquine, doxycycline, azithromycin, ivermectin, COVID-19

An outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which began in Wuhan, China in December 2019, has rapidly spread all over the world. The World Health Organization characterized the disease caused by SARS-CoV-2 (COVID-19) as a pandemic in March 2020. In the absence of specific treatments for the virus, treatment options are being examined. Drug repurposing is a process of identifying new therapeutic uses for approved drugs. It is an effective strategy to discover drug molecules with new therapeutic indications. This strategy is time-saving, low-cost, and has a minimal risk of failure. Several existing approved drugs such as chloroquine, hydroxychloroquine, doxycycline, azithromycin, and ivermectin, are currently in use because of their efficacy in inhibiting COVID-19.

Chloroquine, a drug known for its efficacy in treating malarial and autoimmune diseases such as rheumatoid arthritis and lupus erythematosus, offers promise in inhibiting SARS-CoV-2. Previous studies have revealed that it potentially has broad-spectrum anti-viral activity by increasing the pH of endosomes and lysosomes, thus preventing the process by which the virus fuses with host cells and subsequently replicates (*1*). Chloroquine is the first drug reported to have efficacy against COVID-19 in clinical studies in China (*2*). Feedback from an international meeting that took place to share experiences related to the prevention and control of COVID-19 highlighted the fact that chloroquine demonstrated significant efficacy in reducing the time to virus-negative conversion and in restabilizing body temperature (*3,4*). Hydroxychloroquine, a more tolerable derivative of chloroquine, has also been found to display potent activity against SARS-CoV-2 *in vitro* (*5*). Clinical studies in China have indicated that hydroxychloroquine can help to reduce the time until body temperature returns to normal, decrease the duration of coughing, and improve lung imaging findings (*6*).

Tetracyclines such as doxycycline, minocycline, and tetracycline are well-known antibiotics in clinical use. Tetracyclines are known to inhibit matrix metalloproteinases (MMPs) through their ability to chelate zinc compounds on MMPs. Several functions of the coronavirus, including replication, are associated with the host MMPs complex. Therefore, the zinc-chelating properties of tetracyclines may be efficacious against COVID-19 in humans (*7,8*). Tetracycline was also reported to inhibit the binding of the SARS-CoV-2 spike protein to angiotensin-converting enzyme 2 (ACE2) (*9*). Doxycycline inhibits the entry and replication of SARS-CoV-2 *in vitro* (*10*). Yates *et al*. reported that four high-risk patients with COVID-19

FOI 26-3132 - Document 5

and comorbid pulmonary disease were successfully treated with doxycycline, the doses and durations of which were 100-200 mg/day and 5-14 days, respectively (*11*). Meyboy *et al*. reported that treatment of COVID-19 with doxycycline (100 mg, *b.i.d*., for 7 days) alleviated shortness of breath, coughing, a fever, and O_2 saturation (*12*).

Macrolides such as erythromycin, clarithromycin, and azithromycin exhibit antibacterial activity, immunomodulatory action, and anti-inflammatory action. Recently, the antiviral action of macrolides has attracted considerable attention (*13*). Azithromycin accumulates within lysosomes, increases their pH, and hampers lysosomal functions allowing viral replication (*13*). In addition, azithromycin blocks the interaction points between SARS-CoV-2 and the ACE2 receptor, preventing SARS-CoV-2 from invading host cells (*13*). Tsiakos *et al*. reported that treatment with clarithromycin (500 mg, *b.i.d*., for 7 days) was associated with early clinical improvement in patients with moderate COVID-19 (*14*). Moreover, Ghiasvand *et al*. reported that three patients diagnosed with COVID-19 who did not respond to initial treatment improved after additional treatment with azithromycin (*15*).

Apart from the aforementioned macrolide antibiotics, ivermectin, a macrolide antiparasitic agent, is also an inhibitor of SARS-CoV-2, with a single treatment causing a ~5,000-fold reduction in the virus at 48 h in cell culture (*16*). The mechanism by which ivermectin inhibits SARS-CoV-2 is thought to be *via* the inhibition of the nuclear import of viral and host proteins. In specific terms, importin (IMP) α/β1, a host protein, is a heterodimer that binds to the SARS-CoV-2 cargo protein and moves it into the nucleus, where the complex falls apart and the viral cargo can reduce the host cell's antiviral response. Ivermectin destabilizes the IMPα/β1 heterodimer, preventing it from binding to viral protein and thus from entering the nucleus. As a result, the inhibition of antiviral responses is likely to be reduced, leading to a normal, more efficient antiviral response (*16*). Ivermectin also inhibits the binding of the SARS-CoV-2 spike protein to ACE2, much like macrolide antibiotics (*17*). Ahmed *et al*. reported that a 5-day course of ivermectin (12 mg, daily) for COVID-19 reduced the duration of the illness (*18*).

Examining multidrug therapy for COVID-19, Gautret *et al*. reported that a combination of hydroxychloroquine (200 mg, *t.i.d*., for 10 days) and azithromycin (500 mg on day 1, followed by 250 mg, daily, for the next 4 days) reduced the viral load to an undetectable level on day 6. Moreover, this combined therapy proved to be superior to hydroxychloroquine monotherapy (*19*). Alam *et al*. reported that a combination of ivermectin (0.2 mg/kg, single dose) and doxycycline (100 mg, daily, for 10 days) was efficacious in viral clearance in patients with mild or moderate COVID-19 (*20*). Procter *et al*. treated outpatients with COVID-19 with at least two agents with antiviral activity against SARS-CoV-2 (zinc, hydroxychloroquine, and ivermectin) and one antibiotic (azithromycin, doxycycline, and ceftriaxone) along with inhaled budesonide and/or intramuscular dexamethasone. Consequently, multidrug therapy of early ambulatory patients (not hospitalized and treated at home) was found to be safe, feasible, and associated with low rates of hospitalization and mortality (*21*). Prasad reported a patient with COVID-19 and pulmonary lesions who recovered after receiving early treatment with ivermectin (6 mg, *b.i.d.*, for 3 days), azithromycin (500 mg, daily, for 5 days), doxycycline (100 mg, *b.i.d.*, for 5 days), and prednisolone (50 mg, daily, for 5 days) followed by dexamethasone (6 mg, daily) (*22*). Apart from the aforementioned multidrug therapy, a combination of ivermectin and azithromycin (*23*), a combination of doxycycline and azithromycin (*24*), and a combination of hydroxychloroquine, ivermectin, and azithromycin (*25*) are proposed treatments to be studied in clinical trials.

Multidrug therapy is more efficacious than single-drug therapy because there are differences in the mechanisms of action of the drugs described. Moreover, multidrug therapy may prevent the emergence of drug-resistant SARS-CoV-2. Clinical trials need to be conducted to better assess the efficacy and tolerability of the aforementioned multidrug therapy before it can be adopted on a wider basis.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received January 17, 2021; Revised January 29, 2021; Accepted February 15, 2021.

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Released online in J-STAGE as advance publication February 19, 2021.

FOI 26-3132 - Document 5

TIMELINE — TROPICAL INFECTIOUS DISEASES

The life and times of ivermectin — a success story

Satoshi Ōmura and Andy Crump

Abstract | Since its introduction more than 20 years ago, ivermectin has proved to be one of the most successful therapeutic drugs in veterinary medicine, as well as the basis of one of the most successful public-health programmes of the past century. The drug arose from a unique international collaboration between the public and private sectors. The development process also incorporated the world's first and largest drug-donation programme and involved a unique association between governments, non-governmental organizations and industry. The drug is now being used, free of charge, in two global disease-elimination programmes that are benefiting millions of the world's poorest people.

In 1973, a research partnership was established between the Kitasato Institute in Japan and the Merck, Sharpe and Dohme (MSD) research laboratories in the United States. The outcome of this alliance led to an important advance in animal health products, through the development of an extremely safe drug with a broad spectrum of antiparasitic activity — a drug that has also provided significant benefits to human health for more than 20 years, improving the lives of hundreds of millions of the world's poorest and worst afflicted in the process (TIMELINE).

The partnership

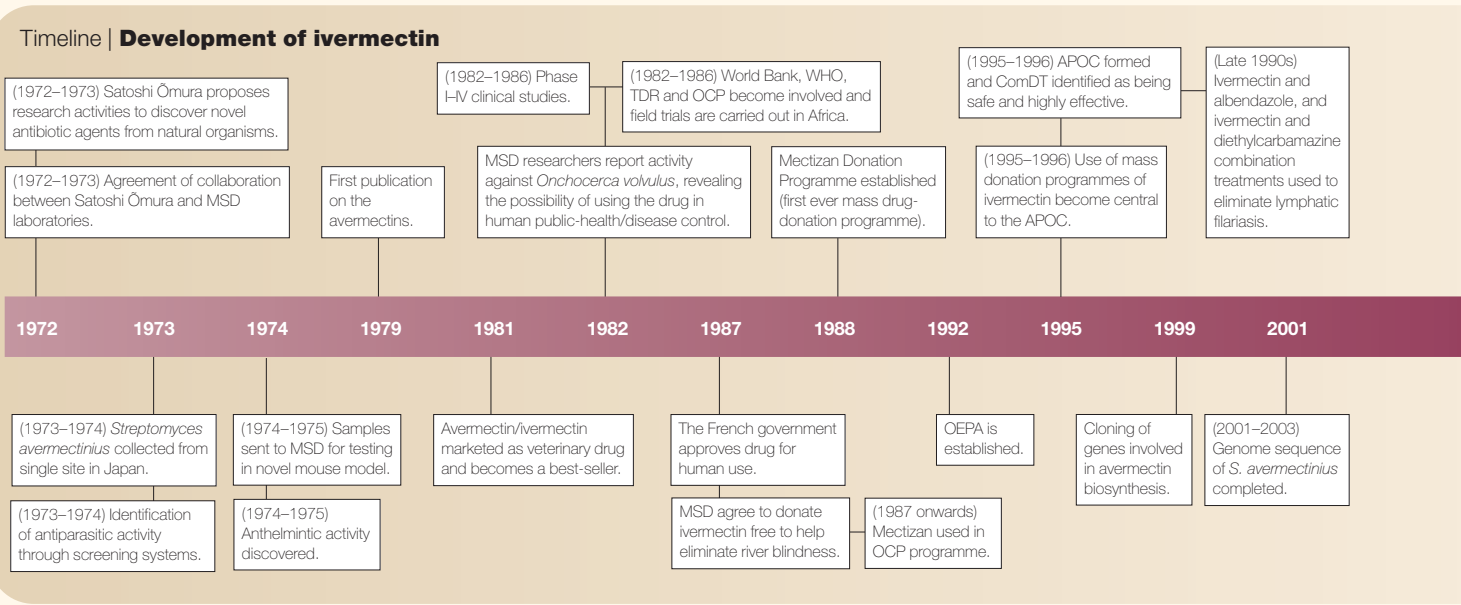
For most of the important breakthroughs in medical research, serendipity has an important role, and so it proved in the case of the drug ivermectin. Researchers at the Kitasato Institute in Tokyo had established a worldwide reputation for their expertise in detecting bioactive compounds produced by microorganisms that are mainly found in the environment. Novel techniques were developed and screening systems were established to increase the chances of accurately detecting minute samples of active compounds[1]. In 1971, one of the authors (S.O.) took a sabbatical from the Kitasato Institute to take up a research post working with Max Tishler, who, after a successful career at MSD, had moved from industry to Wesleyan University in the United States. The Kitasato team were eager to find an industrial partner to participate in a new line of research to discover microorganisms that produce potentially useful compounds with unique chemical structures. Researchers at MSD routinely carried out extensive and innovative *in vivo* work (thereby complementing the *in vitro* work done in Japan) and had the resources to push promising compounds through the drug-development pipeline. By the early 1970s, the testing of synthetic chemicals at the MSD commercial research laboratories was beginning to realize diminishing returns[2], so the company was keen to explore and take advantage of the new research directions that were proposed by the Japanese group. Consequently, the link between Ōmura and Tishler, with his connections to MSD, formed the basis for the new Kitasato–MSD collaboration, which was formally created in 1973.

When the partnership was founded, only a few of the several thousand active fermentation products that had been isolated had anthelmintic activity. Compounds of significance included anthelmycin, anthelvencin, the antibiotic complex S-15-1, aspiculomycin, the axenomycins, G-418, hygromycin B, the destomycins, myxin, paromomycin and the thiamycins. Only one compound, hygromycin, was considered suitable for further development[3]. To address this shortage, the new, goal-oriented research initiative was focused towards discovering a drug that would be effective against helminth parasites[2].

Discovery and isolation

All of the cultures sent from Japan under the terms of the research partnership were from soil samples that were collected and processed by well-established and sophisticated screening systems. These procedures established the morphological features and physiological properties of organisms and compounds produced in fermentation broths and *in vitro*

Timeline | **Development of ivermectin**



evaluations of the chemical products were carried out. In the second year of the collaboration, the Kitasato team isolated an organism — *Streptomyces avermitilis*, which is a species of actinomycete (FIG. 1) — from soil near a golf course bordering the ocean at Kawana, near Ito City in the Shizuoka region. (In 2002, the Kitasato research group published morphological, physiological, biochemical and phylogenetic evidence arguing for the reclassification of the original microorganism and rename it *Streptomyces avermectinius*[4]). The isolated culture was sent, together with 53 other promising microbial samples, to the MSD laboratories in 1974. MSD researchers screened the samples in a novel mouse model of helminth infection in which mice were infected with the nematode worm *Nematospiroides dubius*. Originally identified as OS-3153, the most promising microbial sample was a *S. avermectinius* strain that was shown to have potent anthelmintic activity with little or no toxicity. The compound responsible for the activity was named avermectin[3] (FIG. 2). Further studies demonstrated that avermectin had biocidal activity against a diverse range of nematodes, insects and arachnids. Moreover, the mode of action of avermectin was both unique and robust, and was 25 times more potent than all currently available anthelmintics. The unique ability of avermectin to kill both ecto- and endoparasites gave rise to the new class of compounds called 'endectocides'. In 1979, the first papers on avermectin and its derivatives were published, describing the molecules as a series of macrocyclic lactone derivatives that lacked antibacterial and antifungal activity but which had powerful anthelmintic properties[5–7].

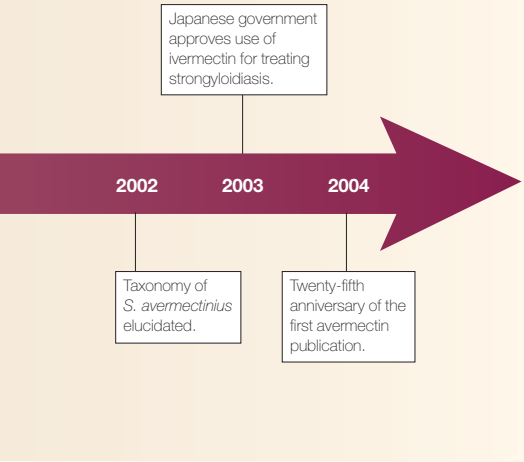




Figure 1 | **Micrograph of *Streptomyces avermectinius*.** *S. avermectinius* is the only organism that has been found to produce the avermectins.

Avermectins are a complex of 16-membered macrocyclic lactones, and fermentation of *S. avermectinius* produces a mixture of eight avermectin compounds (A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a} and B_{2b}) (FIG. 2). Compounds of the B-series containing a 5′-hydroxyl group are markedly more active than those of the A-series, which contain a 5′-methoxyl group. The 1-series and 2-series have similar activity against many parasites[8]. An interdisciplinary team at MSD, headed by William Campbell, investigated the eight active compunds, of which avermectins B_{1a} and B_{1b} were found to have the highest activity. Reduction of the C_{22}–C_{23} double bond of B_{1a} and B_{1b} compounds with Wilkinson's catalyst improved both the spectrum of activity and safety (resulting in very low mammalian toxicity), and the resulting 22,23-dihydro B_1 complex (as a mixture of 80% B_{1a} and 20% B_{1b}) was selected for further commercial development under the generic, non-proprietary name, ivermectin[9]. Although structurally similar to macrolide antibiotics and antifungal macrocyclic polyenes, the avermectins have no antibacterial or antifungal activities.

Further analysis revealed that ivermectin was highly efficacious against mite, tick and botfly ectoparasites, which are organisms that cause massive economic losses in the livestock industry. MSD researchers also observed that the compound had remarkable activity against endo- as well as ectoparasites in horses, cattle, pigs and sheep. Ivermectin was also found to be successful in treating larval heartworms, but not adult worms, and could be used to treat mange and other conditions in dogs. However, no activity was found against flatworms, protozoa, bacteria or fungi[10].

In 1999, the Kitasato group reported that 17 genes of *S. avermectinius* encode enzymes that are involved in avermectin biosynthesis. Of these, those encoding four types of I polyketide synthases are concerned with lactone formation, via 12 cycles and 53 steps. The remainder act on pathway-specific regulation, with 12 genes being involved in modification of the lactone ring, biosynthesis of oleandrose and its glycosylation[53].

In 2003, the Kitasato team sequenced the *S. avermectinius* genome, which is the largest bacterial genome sequence reported so far[11,12]. Analysis of the genome allowed identification of the gene cluster involved in secondary metabolite production, shedding light on the biology of microbial secondary metabolite synthesis at the genetic level.

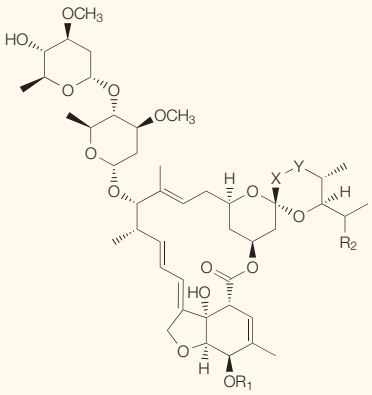
Mode of action

Initially, owing to its rapid and specific antiparasitic and anthelmintic action, it was proposed that ivermectin was an agonist for neurotransmitter function. Experimental studies confirmed this proposal when it was shown that inhibition occurred via glutamate-gated chloride ion channels in nerve and muscle cells[13]. Ivermectin interacts with these channels, preventing their closure. Consequently, synapse membranes become increasingly permeable to chloride ions, which leads to hyperpolarization of the neuronal membrane and decreases, or prevents, neuronal transmission. This, in turn, leads to paralysis of the somatic muscles, particularly the pharyngeal pump, causing the death of the parasite[14,15]. γ-aminobutyric acid (GABA)-related chloride ion channels, which are present only in nematodes, insects and ticks, are only inhibited with greater drug concentrations[16–18]. In mammals, GABA receptors and neurons are found in the central nervous system, whereas in arthropods and nematodes they are located in the peripheral nervous system. This, coupled with the relatively low dose concentrations needed, ensures that mammals can ingest ivermectin with a high degree of safety.

Animal health

Ivermectin was introduced to the market in 1981 as a veterinary antiparasitic drug and soon proved to be the most effective, broad-spectrum antiparasitic drug ever developed. It quickly became a remarkable success, rapidly capturing a large portion of the global veterinary antiparasitic market, and became the market leader within two years — the drug has maintained that position ever since, with annual sales of about US$1 billion. Since the introduction of ivermectin, several other

FOI 26-3132 - Document 5



	R_1	R_2	X–Y
Avermectin A_{1a}	Me	Et	CH=CH
Avermectin A_{1b}	Me	Me	CH=CH
Avermectin A_{2a}	Me	Et	$CH_2CH(\alpha OH)$
Avermectin A_{2b}	Me	Me	$CH_2CH(\alpha OH)$
Avermectin B_{1a}	H	Et	CH=CH
Avermectin B_{1b}	H	Me	CH=CH
Avermectin B_{2a}	H	Et	$CH_2CH(\alpha OH)$
Avermectin B_{2b}	H	Me	$CH_2CH(\alpha OH$

Figure 2 | **Structure of the avermectins.** The inclusion of different groups at positions R_1, R_2, X and Y create the eight different avermectin compounds A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a} and B_{2b}.

endectocides have appeared but none have replaced ivermectin as the market leader.

Five years after its introduction, ivermectin was registered for use in 46 countries and was being used worldwide to treat approximately 320 million cattle, 151 million sheep, 21 million horses and 5.7 million pigs[19]. Most horses in the United States were treated with the drug to the extent that the horse nematode *Onchocerca cervicalis* has now virtually disappeared.

Public health

At the time the collaboration between the Kitasato Institute and MSD was being set up and becoming operational in the mid-1970s, onchocerciasis (or river blindness) had long been a major global health problem. The disease results from infection with *Onchocerca volvulus*, which is a parasitic worm that lives in the human body. Adult female worms live for up to 14–15 years, producing millions of microscopic larvae (microfilariae) that migrate in the body, causing a variety of symptoms (FIG. 3). The bite of infected blackflies (*Simulium* spp.) transmits immature larval forms of the worms from human to human. Adult worms, which take a year to mature, lodge in nodules under the skin, releasing millions of microfilariae into surrounding tissues, which then migrate through the body, living for 9–18 months. When the microfilariae die, the resulting residue causes visual impairment and blindness, skin rashes, lesions, intense itching and depigmentation of the skin, lymphadenitis and general debilitation. Onchocerciasis is found in 35 countries, including 28 nations in tropical Africa where 99% of infected people live (FIG. 4). Isolated foci also occur in Latin America and Yemen. Each year, approximately 18 million people are infected, with more than 6.5 million people suffering from severe itching, dermatitis and impaired visual acuity, and 270,000 patients suffering from complete blindness. The disease is estimated to be responsible for the loss of 1 million disability-adjusted life years (DALYs) annually. In the early 1970s, the only drugs that were available to treat the disease were suramin, which is a highly toxic molecule that had to be administered in repeated injections over a period of several weeks, and diethylcarbamazine, which also had to be administered over several weeks. Diethylcarbamazine can also cause severe side effects due to the accumulation of dead parasitic tissue, a process that can result in blindness. By the mid-1970s, it was clear that both drugs could actually worsen eye lesions and so the use of chemotherapy for onchocerciasis was stopped[20].

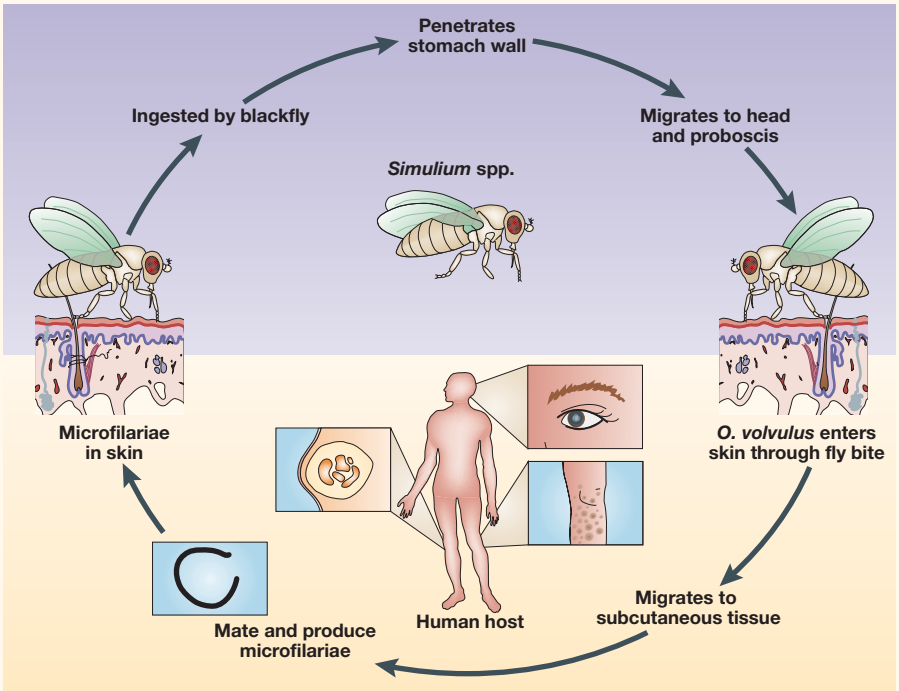


Figure 3 | **Schematic representation of the life cycle of *Onchocerca volvulus*.** When a parasitized female blackfly (*Simulium* spp.) takes a blood meal, infective *Onchocerca* larvae pass into the host through the fly-bite wound. Larvae enter subcutaneous tissue, where they migrate, aggregate in nodules, mature into adult worms and mate. After mating, eggs in female worms develop into immature larvae (microfilariae), each worm is capable of producing 1,000 microfilariae per day. Many thousands of microfilariae are released to migrate in subcutaneous tissue. When they die, they cause skin rashes, lesions, intense itching and skin depigmentation. Microfilariae also migrate to the eye and can cause visual impairment and blindness. When the infected host is bitten by another female fly, microfilariae pass into the blackfly, where they develop into infective larvae, and the life cycle continues.

There was hope, however, for a new and effective treatment. The MSD research in horses had demonstrated that ivermectin killed *Onchocerca cervicalis*, which are nematodes that are closely related to *Onchocerca volvulus*, so work began to investigate the possible use of ivermectin in humans. MSD joined forces with the World Health Organization (WHO), the Special Programme for Research and Training in Tropical Diseases (TDR) and the Onchocerciasis Control Programme in West Africa (OCP) to carry out an extensive collaborative research programme in humans. This proved to be an early example of the so-called public–private partnerships (PPPs) that are now being created in increasing numbers to coordinate the production and distribution of effective therapies for diseases for which there is little likelihood of a financial return on investment.

In 1981, clinical trials of ivermectin, which was produced under the brand name 'Mectizan', began in Senegal[21,22]. Over the next

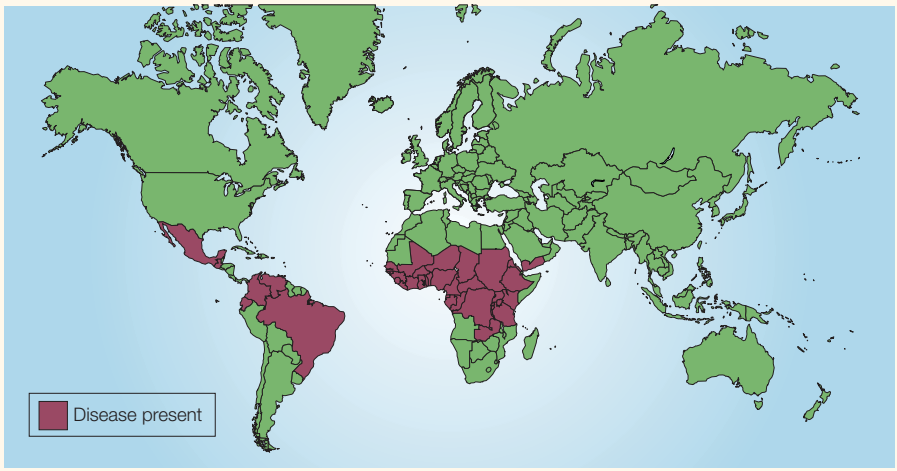


Figure 4 | **Global distribution of onchocerciasis.** Data obtained from the WHO.

four years, large-scale field trials involving hundreds of thousands of individuals were undertaken in Ghana, Guatemala, Cote d'Ivoire, Liberia, Mali, Senegal and Togo. Phase II studies reported that local inflammatory responses in ocular tissue were less severe with ivermectin than with diethylcarbamazine, thereby avoiding the unwanted Mazzotti reaction — by which the accumulation of dead parasitic material can induce blindness. Results showed that a single annual dose of 200 μg per kg of body weight eradicated microfilarial worms from the eye and skin after one month, and patients remained worm-free for up to 12 months after treatment[23–25]. Later, large-scale community trials in various epidemiological conditions, including hyper- and meso-endemic areas, found that ivermectin reduced the levels of skin microfilaria by 96–99% within the first few months of therapy[26]. It has been repeatedly shown that ivermectin has little impact on adult worms and that multiple doses do not have much impact on oogenesis[27]. Adult worms can continue to reproduce, so ivermectin is suppressive rather than curative; however, treatment leads to a marked reduction in the number of immature worms and reduces microfilarial loads after each course of treatment[21,28]. Several follow-up studies have shown that the marked reduction in both prevalence and intensity of microfilaridermia is maintained, even after five years of treatment[29–31]. Ivermectin has the added benefits of allowing the body to repair minor eye lesions and reduces the severity of itching and the prevalence of skin lesions[32]. Some researchers argue that ivermectin alters the mechanisms by which adult worms locate each other and that this explains why fewer males are found in nodules after multiple treatments[33]. More frequent administration of ivermectin, for example bi-annually rather than annually, could have a sterilizing effect on adult female worms and reduce their lifespan[34]. Nevertheless, taking the drug over the lifespan of the adult worms effectively prevents patients from developing the disease.

Following the work of a truly international partnership involving the public and private sectors, governments of disease-endemic countries and affected communities, the human formulation of ivermectin was registered for use by French regulators in 1987 with mass drug administrations commencing in 1988.

The use of ivermectin can cause adverse side effects, the most common being oedema, fever, pruritus and pain[35,36]. They are generally mild and transient, however, and can be easily treated by primary healthcare workers or trained lay members of the community. Indeed, the incidence of adverse reactions in follow-up studies on distribution programmes has actually been less than that observed during clinical trials[37]. Ivermectin can also have serious effects on individuals heavily infected with *Loa loa*, which causes severe encephalopathy that can prove fatal[38]. Consequently, it is essential to take appropriate safety measures to exclude any such people from mass drug-administration programmes. A comprehensive review of serious adverse events following Mectizan treatment carried out in 1989–2001 found 207 cases from a reported 165 million treatments, representing an incidence ratio of 1 per 800,000 treatments[39].

Drug donation

During the latter stages of human trials, discussions took place between the public and private sectors to determine a suitable price for the drug, bearing in mind that the end-users of the product would be poor and marginalized communities. In 1987, after receipt of consent from the Kitasato Institute, which agreed to forego royalties, P. Roy Vagelos, the then chief executive of MSD, announced that ivermectin (under the brand name Mectizan) would be provided free of charge for the treatment of river blindness for "as long as it is needed", a pledge that has been honoured ever since. The advent of this drug donation has transformed the treatment of onchocerciasis to a point where elimination of the disease has become an achievable goal.

Community-directed treatment

Also in 1987, the TDR began to fund pioneering research on the concept of 'community-based treatment' using ivermectin. The rationale behind this concept was that the drug was so safe and easy to use that affected communities should be able to organize their own drug distribution and treatment. In the field, community-directed treatment proved to be remarkably successful and, in 1989, the WHO announced that ivermectin could be administered "with minimal supervision". It was subsequently noted that ivermectin coverage was greatest when affected communities were allowed to design and implement their own drug-distribution programme[40]. The TDR went on to develop the community-directed treatment with ivermectin (ComDT) procedure, which became the operational basis of the African Programme for Onchocerciasis Control (APOC). Established in 1995, the aim of APOC is to create sustainable community-directed distribution systems using mass distribution of ivermectin to all those eligible by 2007, ultimately covering 59 million people in African countries where the disease remains a serious public-health problem and where 15 million people are heavily infected. The goal is to eliminate the disease in the 17 African nations where it still persists after the successful control of the disease in the 11 west-African nations covered by the OCP. Meanwhile, the Onchocerciasis Elimination Programme in the Americas (OEPA), which was launched in 1992, is based on the premise that mass administration of Mectizan tablets can rid the region of the disease relatively quickly, as the vector blackflies in this region transmit the parasite with reduced efficiency. The programme is expected to achieve its goal of eliminating the disease from the region by 2007 (REF. 20). Community-directed treatment is producing excellent results in the fight against onchocerciasis. Affected villages collect Mectizan tablets, deliver them to all those eligible and report back to health authorities — and meet all the costs involved themselves

FOI 26-3132 - Document 5

(the drug being provided free to district health posts). The correct dosage can be calculated simply by measuring the height of the recipient. In 2003, approximately 56 million Africans were taking a single annual dose of ivermectin[41]. The overall goal of these programmes is to eliminate onchocerciasis as a public-health problem globally by 2010.

Economic aspects

Mectizan is viewed by some as one of the finest public-health success stories of the past century, and a model for all future public and private sector partnerships. However, analysis of the economics indicates that success depends on financial commitment from both sectors. Cost-effective analyses of the mass distribution of ivermectin have identified a cost of US$14–30 per DALY that is prevented[42]. This compares reasonably well with other control programmes for priority diseases, but the benefit ratio in this case is only possible due to the huge financial commitment from Merck & Co., which pays the production costs of Mectizan, the costs of transport from the manufacturing facility in France to the recipient countries and the related customs and other handling costs. In total, more than 250 million doses of ivermectin have been administered since the start of the Mectizan donation programme. In 2000, APOC devoted 63% of its total annual budget of US$9.4 million to ivermectin distribution programmes. Using the US$1.50 figure claimed by the Mectizan Donation Programme as being the cost of a single tablet, the value of the ivermectin contributed by Merck & Co. in 2001 is estimated to be US$143.6 million for APOC countries alone. This can be compared with the total 15-year costs of the APOC programme, which are estimated to be US$182.5 million[43].

APOC and others are working towards reducing the actual costs of 'free drug' donation programmes, with APOC focusing on a target distribution cost of US$0.20 per treatment[43]. A study of two villages in Nigeria found that 92–93% of inhabitants were willing to pay US$0.30 and US$0.28, respectively, to meet their ivermectin distribution needs. The actual costs of treatment in the two villages were calculated as US$0.13 and US$0.17, respectively[44,45], which bodes well for maintaining sustainable, long-term mass distribution programmes for the period of time that is needed to achieve disease elimination. The continuing, concerted and long-term commitment of donors (led by the World Bank), non-governmental organizations, governments, health services and affected communities will be essential if the elimination goal is to be achieved.

Lymphatic filariasis

The success of ivermectin does not stop with the potential elimination of onchocerciasis as a public-health threat. Combinations of ivermectin and albendazole or ivermectin and diethylcarbamazine have been adopted as the basis of mass treatment in the Global Alliance to Eliminate Lymphatic Filariasis, which was initiated by the WHO in 1997. The efficacy of the ivermectin and albendazole combination therapy for bancroftian filariasis was first reported in 1997 (REF. 46). Lymphatic filariasis is one of the most prevalent tropical diseases, with an estimated 120 million people being infected annually. The disease is responsible for 5 million DALYs lost each year, ranking it third among tropical diseases in terms of DALYs, after malaria and tuberculosis. Almost 1 in 5 of the world population are at risk of infection and the disease remains a major impediment to socio-economic development in endemic areas — India loses an estimated US$1 billion annually as a result of lymphatic filariasis. In an effort to curb this disease, Merck & Co. confirmed that they would donate Mectizan free of charge to the elimination programmes in regions where onchocerciasis and lymphatic filariasis coexist. GlaxoSmithKline, the manufacturers of albendazole, also announced that they would donate this drug for lymphatic filariasis control programmes, which are working towards the goal of eliminating the disease by 2020.

New uses for ivermectin continue to be found. For example, in 2003, ivermectin was registered for the treatment of strongyloidiasis, an intestinal parasitic disease that is widely distributed in the tropics and subtropics and which is prevalent in south-east Asia and southern Japanese islands. More than 10 million people are infected with the causative microorganism.

Future use

A microorganism from a single site in Japan has provided a drug that has proved to be one of the most successful tools in human and animal health, and one that has been the mainstay of several of the most successful disease-control programmes in the history of public health. Moreover, the benefits continue to accrue. It is common knowledge that malnutrition and undernutrition are major causes of ill health in Africa and throughout the developing world, particularly among young children. Ivermectin is known to be particularly effective against infection with *Ascaris* worms and reasonably effective against hookworm and *Trichuris*[47,48]. Furthermore, the prevalence of head lice is markedly reduced in children taking ivermectin tablets[49]. In Japan, ivermectin may soon be routinely used in special circumstances, such as in homes for the elderly to help control the incidence of scabies.

Perhaps of greater significance, ivermectin became available at a time when resistance to other similar drugs, such as benzimidazole and levamisole, was common. Very few cases of resistance to ivermectin have been reported and any future development of widespread resistance is thought to be unlikely[16]. Parasites that are resistant to the avermectins are also resistant to the milbemycins and it is likely that P-glycoproteins (plasma-membrane-associated drug efflux transporters) are involved in the resistance mechanism[50]. It is also postulated that resistance could be mediated by mutations in the gene encoding the α-subunit of glutamate-gated chloride channels; However, the exact resistance mechanism is still a matter of conjecture[51].

The discovery, development and deployment of ivermectin through the efforts of Merck & Co. and the Kitasato Institute, aided by a group of international partners from a range of disciplines, has been hailed by some commentators as one of the greatest medical achievements of the twentieth century[52]. Vigilance is now needed to ensure that the progress that has been made against several devastating diseases is maintained. The strain of *Streptomyces* found in Japan remains the only microorganism that produces avermectin. The Kitasato Institute, and similar organizations, remain dedicated to the search for further microbiological treasures for use in public health from among the myriad of substances produced by the world's reservoir of environmental microorganisms.

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doi:10.1038/nrmicro1048

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Competing interests statement
The authors declare no competing financial interests.

Online links

FURTHER INFORMATION
WHO: http://www.who.int/en/
TDR: http://www.who.int/tdr/
Onchocerciasis control programme: http://www.who.int/pbd/blindness/onchocerciasis/ocp/en/
African programme for onchocerciasis control: http://www.who.int/pbd/blindness/onchocerciasis/apoc/en/
Global alliance to eliminate lympatic filariasis: http://www.filariasis.org/index.pl
CDC onchocerciasis fact sheet: http://www.cdc.gov/ncidod/diseases/submenus/sub_river_blindness.htm
CDC lymphatic filariasis fact sheet: http://www.cdc.gov/ncidod/diseases/submenus/sub_filariasis.htm
Access to this links box is available online.

OPINION — ANTI-INFECTIVES

Antibiotic resistance: a view from the prescriber

Roger G. Finch

Abstract | Antibiotic resistance is increasingly affecting the management of infectious diseases. The prescribing clinician is not only important to the development of the problem but also central to its solution. This article addresses the current weaknesses in the information systems and the evidence base that support prescribing. Remedies necessary for improvements in prudent prescribing include better guidance in managing specific diseases where resistance is of prognostic significance and also increasing diagnostic precision.

The increasing prevalence of antibiotic-resistant microorganisms is now an issue of major public concern. Newspaper headlines and media reports regularly feature details of their impact on individuals as well as raising the spectre of widespread and untreatable drug-resistant infections. The breadth and significance of antibiotic-resistant micro-organisms is becoming increasingly apparent. Examples include outbreaks of multiply antibiotic-resistant pathogens in intensive care or neonatal units; the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and, more recently, nursing homes; the global occurrence of drug-resistant malaria in tropical and sub-tropical regions; and drug-resistant tuberculosis, with its links to poverty, HIV and social exclusion.

Reports on antibiotic resistance and concerns raised by infection specialists over the past fifty years were largely unheeded until the mid-1990s when the significance of the problem achieved political recognition. In 1995, the American Society for Microbiology[1], and in 1998, a House of Lords Select Committee in the United Kingdom[2], issued reports that have been influential in establishing national strategies. In the United States, a task force was established[3]. The European Union also published its strategy[4] and addressed the problem through a series of conferences on antibiotic resistance, such

FOI 26-3132 - Document 5

International Journal of Advanced Research in Medicine

E-ISSN: 2706-9575
P-ISSN: 2706-9567
IJARM 2021; 3(2): 05-09
Received: 04-03-2021
Accepted: 09-06-2021

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A comparative study on ivermectin- doxycycline and favipiravir therapy in COVID19 patients

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DOI: https://doi.org/10.22271/27069567.2021.v3.i2a.207

Abstract
Objectives: We investigated the outcomes of Ivermectin-Doxycycline vs. Favipiravir therapy in mild to moderate COVID19 patients.
Methods: Patients were divided randomly into two groups: Ivermectin 200µgm/kg single dose + Doxycycline 100mg BID for ten days in group A, and Favipiravir 1800mg for the first day, then 800mg BID for six days in group B (Control group). RT-PCR for SARS-CoV-2 infection was repeated in all symptomatic patients on the second day onward without symptoms. Repeat PCR was done every two days onward if the result found positive. Time to the negative PCR and symptomatic recovery was measured for each group.
Results: All subjects in Group A reached a negative PCR, at a mean of 8.93 days, and reached symptomatic recovery, at a mean of 5.93 days, with 55.10% symptom-free by the fifth day. In group B, 96.36% reached a negative PCR at a mean of 9.33 days and were symptoms-free at 6.99 days. In group a 31.67% of patients expressed symptoms caused by medication, this was 46.43% in group B.
Conclusion: The combination therapy of Ivermectin-Doxycycline showed a trend towards superiority to the combination of Favipiravir for mild to moderate COVID19 disease.

Keywords: COVID-19, doxycycline, favipiravir, ivermectin

Introduction
Coronavirus disease 2019 (COVID-19) is a global pandemic declared by the world health organization (WHO). Over ninety million people have already been infected by severe acute respiratory syndrome- coronavirus- 2 (SARS-CoV-2), and billions have been affected by the socioeconomic squeal. As SARS-CoV-2 is a novel virus, there are no proven treatment options yet. Early treatment before the disease becomes severe would be optimal. Recently, an anti-parasitic drug Ivermectin has been described as highly effective in an *in vitro* study against SARS-CoV-2 [4]. an encouraging result in a case series of COVID19 patients with a combination of Ivermectin and Doxycycline. Ivermectin is well-tolerated, less toxic, and has fewer adverse effects than Favipiravir.

Methods
Informed Consent was taken. The individuals who gave consent were enrolled in this study. Study Population and Data Collection Patients (16 years to 80 years of age) tested positive for SARS-CoV-2 infection by Real-time polymerase chain reaction (RT-PCR) at Geetanjali Medical College and Hospital from May 2020 to the December 2020, were initially included in this study, including those with and without the symptoms. All patients received a full evaluation, including a history of current illness, comorbid condition, and associated complaints. Patients with unstable comorbid conditions like bronchial asthma, chronic obstructive pulmonary disease (COPD), ischemic heart disease (IHD), uncontrolled diabetes mellitus (DM), advanced renal and hepatic disease, carcinoma, hospitalized, and immuno-compromised patients were excluded. Patients were examined for oxygen saturation and only those with oxygen saturation of 95% or above who fit the outpatient treatment protocol for COVID19 were included. Patients with respiratory symptoms received chest radiographs. Those with normal or near-normal chest radiographs (up to 10% involvement) were

included. Randomization and Treatment Intervention Randomization was done using an odd-even methodology and applied to registration number of study patient, consecutively in a 1:1 ratio. Treatment was given, and the final enrolment was done by the attending physician (investigator). All the patients enrolled in this study were treated as outpatient department (OPD) patients. For the study, patients were divided into two groups, as follows: • Group A (n=60): Ivermectin 200µgm/kg single dose + Doxycycline 100mg BID for ten days. • Group B (n=56): Favipiravir 1800mg for the first day, then 800mg BID for six days. All the subjects were provided with symptomatic treatment such as fever, headache, cough, and myalgia. Drug interactions and contraindications for each individual were considered carefully. The schedule of medication intake was adequately explained to each patient. Group A's instructions included that Ivermectin tablet (200µg/ kg) single dose to be taken on an empty stomach an hour before a meal on the first day. Doxycycline 100 mg capsule be taken twice daily after meal for ten days starting from day one. Group B's instructions included Favipiravir 1800mg for the first day, then 800mg twice daily after meal for six days. Patients were advised to self-isolate, take proper nutrition, hydration, and maintain a sanitary environment. Repeat Nasopharyngeal and Throat Swab PCR All subjects underwent repeat nasopharyngeal and throat swab PCR for SARS-CoV2 every other day until their PCR was negative. These repeat PCR tests began on the fifth day after taking the medication for subjects who began the study and remained symptom-free. The PCR repeat testing began on the second symptom-free day onward for subjects who began the study with symptoms or developed symptoms. The investigators had telephone contact with all the subjects every three days throughout the study to determine any therapy's adverse effects. A re-evaluation PCR was performed after seven days following the first negative PCR.

Data Analysis and Statistics: Data were presented as mean±standard deviation, and statistical analysis was done by Graph pad Prism software. Column analysis was done to find the mean with the standard deviation in each group. T-test was done to see the significance between the values where needed. P>0.05 was considered statistically significant.

Results

181 patients tested positive for SARS-CoV-2 infection in that period. 42 patients had comorbid conditions (some required hospitalization) that might have affected the recovery time, and 14 patients were unwilling to participate in the study. Nonetheless, 9 patients did not show-up (3 from group A and 6 from group B) for the follow-up sample collection, so these were excluded, and 116 patients were finally included in the analysis. Demographic Characteristics of the Study Subjects As shown in Table 1, the total number of patients was 116; male 84 and female 26, age 16 to 80 years, and mean age (33.94±14.12 years). Group A (Ivermectin + Doxycycline): male 43 (71.67%), female 17 (28.33%), age 35.72±15.1 years; males 37 years and female 32.88 years. Group B (Favipiravir): male 47 (83.93%), female 9 (16.07%), age 31.91 years; male 31.35, and female 34.5 years. Among the total, 91 (78.45%) were symptomatic, and 25 (21.55%) were asymptomatic patients with contact history. These were 49 (81.67%) and 11 (18.33%) in group A, 42 (75%) and 14 (25%) in group B. Recovery Rate and Mean Recovery Duration Between Groups In group A, recovery to negative PCR rate was 100% (60/60). The mean recovery duration to negative PCR was 8.93 days (8 to 13 days). 41 (63.3%) of patients had no new complaints other than their presenting symptoms. New symptoms that may have been attributed to adverse drug effects included lethargy in 14 (23.3%), nausea in 11 (18.3%), and occasional vertigo in 7 (11.66%) of patients (Fig. 1c). In group B, out of 56 patients, two male patients were shifted to ICU. They did not recover to a negative PCR as part of the study. Therefore, the recovery rate to negative PCR was 96.36% (54/56). The mean duration of recovery to negative PCR was 9.33 days (5 to 15 days). 30 (53.57%) of the patients had no new complaints other than their presenting symptoms. Fresh symptoms that were recognized as an adverse effect of HCQ included 13(23.21%) with mild blurred vision and headache; 22 (39.2%) with increased lethargy and dizziness, 10 (17.85%) with occasional, mild palpitation, and 9 (16.07%) with nausea and vomiting. Difference in Recovery to Negative PCR between Groups The difference between group A and group B recovery to negative PCR duration was not statistically significant in unpaired t-test, p=0.2314. Subgroup analysis of the recovery duration: male 9.18±1.90 days and female 8.92±1.32 days, p=0.515; in group A male 8.907±1.342 days and female 9±1.173 days, p=0.44; and in group B male 9.18±1.90 days and female 8.92±1.32 days, p=0.407. The recovery duration of both group males and females were not significant, p=0.18 and 0.69, respectively. The mean duration of symptomatic recovery was 5.93 days (5 to 10 days) in group A and 6.99 days (4 to 12 days) in group B. This difference in time to symptomatic recovery between group A and group B is not statistically significant, p=0.071. In group A, over half of the subjects had become symptom-free by five days 27 (55.10%), with the remaining subjects becoming symptom-free on day six (16.32%), day seven (12.24%), day eight (8.16%), day nine (4%), and day ten (2.04%). In group B, recovery was slower with subjects becoming symptoms free on fourth day 3 (7.14%), the fifth day 10 (23.8%), sixth day 9 (21.43%), seventh day 8(19.04%), eighth & ninth day 4 (9.52% each), eleventh day 2 (4.76%), and tenth and twelfth day (2.38% each). Mean Duration of Time to Negative PCR between Groups In the secondary analysis of subjects who began the study with symptoms, the mean duration of time to negative PCR was 9.061 days in group A and 9.738 days in group B. This was not statistically significant in the unpaired t-test, p=0.0714. The mean duration of time to becoming negative PCR of patients without symptoms was 8.364 days in group A and 7.917 days in group B, which was not statistically significant in unpaired t-test, p=0.443. Further analysis showed the highest recovery was achieved on the eighth day among group A patients in case of both asymptomatic (n=11) and symptomatic (n=49) patients, 8 (72.72%) and 22 (44.89%), respectively (Fig. 2 c, d). This recovery was relatively slower in group B. On the sixth day 3 (7.5%), seventh day 1 (2.5%), eighth day 9 (22.5%), ninth and tenth day 8 (20%) each, eleventh and twelfth day 4 (10%) each, thirteenth day 1 (2.5%), and fourteenth day 2 (5%) in group B patients with symptoms (n=40). Among asymptomatic patients (n=14), this was 1 (7.5%) on the fifth day, 2 (14.2%) individually on the sixth, seventh, eighth, tenth day, 4 (28.57%) on the ninth day, and 1 (7.1%) on the eleventh

day. No significant difference in the recovery duration was found in the subgroup analysis of the recovery duration according to the study group's age. 61 to 70 years in group A had the longest recovery duration, 9.5±2.12, and 71 to 80 years was the shortest 8 days. In group B, this was 11.71±2.48 days in the 41 to 50 years and 8.37±2.44 days in the 10 to 20 years age group.

Table 1: Baseline characteristics of the study group patients.

Parameters	
Number of patients (n)	116
Male	90
Female	26
Group A (n)	60
Group A Male	43
Group A Female	17
Group B (n)	56
Group B Male	47
Group B Female	9
Age (in years)	33.94±14.12 (8 to 80 Years)
Symptomatic	91 (78.45)
Asymptomatic (n, %)	25 (21.55)

Table 2: Showing Group A

Age group A (in years)	35.72±15.1
Male	37
Female	32
Symptomatic	49
Asymptomatic	11

Table 3: Showing Group B

Age group B (in years)	31.91±12.72
Male	31
Female	34
Symptomatic	42
Asymptomatic	14

Discussion

The COVID-19 pandemic in India is part of the coronavirus worldwide pandemic disease caused by a newly discovered coronavirus. It was initially called novel coronavirus and later named SARS-CoV-2 due to its similar characteristics with SARS-CoV-1 [7-9]. New concerns about Favipiravir has led us to seek alternatives with shorter recovery time and better tolerability. Thus, we have undertaken a comparative therapeutic analysis, comparing these standard drugs with Ivermectin and Doxycycline. In this treatment study of groups A and B, the presenting symptoms of the COVID19 patients were fever, cough, sore throat, weakness, chest discomfort, breathing difficulty, diarrhea, myalgia, and abdominal pain. To avoid the recovery duration's influence, we solely selected the cases devoid of severe comorbidities. The difference in recovery to negative PCR duration was not significant (p=0.231) among the two groups. The mean recovery duration is shorter, 8.933 days in group A than in group B, 9.33 days. Also, group A had a better outcome ratio of 100% (60/60) recovery to negative PCR compared to that of group B 96.36% (54/56). At present, it has been the topic of discussion concerning Favipiravir potential use to treat patients with COVID-19 [11]. It is thought that the effect of Favipiravir results in the selective killing of the infected cells. Therefore, it may accelerate viral clearance in COVID-19 [13]. Some studies showed that severe deterioration in some patients with COVID-19 had been closely associated with dysregulated and excessive cytokine release termed "cytokine storm [14, 15]." Favipiravir was found to inhibit SARS-CoV-2 infection *in vitro* and significantly decrease cytokine production, especially the pro-inflammatory cytokines [16]. On the other hand, Ivermectin is a relatively safe and well-tolerated anti-parasitic drug that can inhibit nuclear transport activity [20]. Recently, *in-vitro* studies have shown its function against SARS-CoV-2 [21, 22]. A report suggested that Ivermectin reduces mortality rates in hospitalized patients with COVID-19 [23]. However, it is unknown if antiviral levels are attainable while using known dosing regimens of Ivermectin therapy in patients with COVID-19. [24, 25] Thus, it is vital to investigate Ivermectin's dose regimens for COVID-19 treatment or to determine if there is appropriate synergism using combination therapy with another drug. Also, Doxycycline is a tetracycline class of antibiotics with a long history of clinical use [26]. The efficacy and tolerability of Ivermectin and Doxycycline were established in combination with an earlier study to treat onchocerciasis [27]. Several recent studies have suggested a therapeutic role of Doxycycline against COVID-19 [28, 29]. In our study, the difference in recovery to become symptom-free was not statistically significant. Nevertheless, the Ivermectin group showed better symptomatic recovery than the Favipiravir group. According to the age among study groups, the difference was not statistically significant. Ivermectin-Doxycycline combination expressed an earlier and faster relief of COVID features and viral clearance than the favipiravir combination. However, the mean recovery duration is not statistically significant. In the Ivermectin-Doxycycline group a greater number of patients gained faster symptomatic recovery than that of the Favipiravir group. This suggests Ivermectin-Doxycycline may have better efficacy in reducing the COVID-19 symptoms than Favipiravir therapy. The Ivermectin-Doxycycline group had better patient compliance and fewer adverse effects compared to the Favipiravir group. The adverse effects of Favipiravir in our study are similar to others [30, 31]. The sex difference was also examined, but there were no significant differences between males and females in this study. According to this study, the Ivermectin-Doxycycline treatment regimen was well tolerated, and effective treatment for mild to moderate degrees of SARS-CoV-2 infection. Not only concerning the time to become symptom-free and the viral clearance, but also in terms of safety, side-effect profile, and compliance the Ivermectin-Doxycycline combination is superior to Favipiravir therapy for mild to moderate degrees of COVID-19 patients. We strongly believe that increasing the dose and the duration of Ivermectin treatment will further benefit in reducing the recovery period of COVID19 infection beyond that which was seen in our study. This will also prevent disease progression and morbidity in COVID-19 patients. Our study has limitations, and these include relatively small sample size, the dose of Ivermectin, and case selection. The outcome may be biased by factors like disease severity, lack of cooperation of some participants, and unknown comorbidity.

Conclusion

According to our study, the Ivermectin-Doxycycline combination therapy has better symptomatic relief, shortened recovery duration, fewer adverse effects, and

superior patient compliance compared to the Favipiravir. Based on this study's outcomes, the Ivermectin-Doxycycline combination is a superior choice for treating patients with mild to moderate COVID-19 disease. Despite this study's limitation, we tried to select our study group patients without any major or unstable comorbid condition as far as possible to avoid differences in treatment outcomes among the groups.

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FOI 26-3132 - Document 5
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ORIGINAL ARTICLES

Comparison of Viral Clearance between Ivermectin with Doxycycline and Hydroxychloroquine with Azithromycin in COVID-19 Patients

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Abstract:

Introduction: The whole world is on the brink of collapse due to the outbreak of COVID-19 with no solution to treat these cases with any specific drug. Extensive search for the Vaccine or effective treatment is going on while alarming infection and death toll is rising every day.

Aim: The study will compare the effect of Ivermectin with Doxycycline and Hydoxychloroquine with Azythromicin on a selective group of COVID -19 positive patients.

Method: This is a comparative study that included 400 patients of COVID 19 positive patient who were divided in to two groups. Group- A Received Ivermectin with Doxycycline and the other group- B received hydroxichloroquine(HCQ) with azithromycin.

Result: Viral clearance is 132 (66%) on day 5 and 167(83.5%) on day 6. Among them 33(16.5%) remain PCR positive after 6th day of Ivermectin ingestion in Group A. Whereas there is154 (77.0%) viral clearance at 11th day and 163(81.5%) viral clearance at 12th day of Hydroxychloroquine ingestion in Group B. Among them 37 (18.5%) remain PCR positive after 12 day in group B. The P value is 0.000427 which is significant considering 5th day viral clearance of Ivermectin ingestion and 11th day of Hydroxychloroquine ingestion. But considering 6th day and 12th day the P-value is 0.59 which is not significant.

Conclusion: It appears Ivermectin and Doxycycline is safeand effective combination drug therapy in COVID-19infected patients but need further extensive study to find out the scope of application on other groups of patients.

Keywords: COVID-19, Ivermectin, Hydroxychloroquine, Doxycycline, Doxycycline Azithromycin Hydroxychloroquine with Azithromycin

(J Bangladesh Coll Phys Surg 2020; 38: 5-9)
DOI: https://doi.org/10.3329/jbcps.v38i0. 47514

Introduction:

While the world is awaiting a vaccine, the death toll is rising day by day. We need to find effective drugs to treat coronavirus disease-19 (COVID-19). Several drugs have been proposed and practiced worldwide but none is confirmed or approved. In this study we are looking for an answer for the large group of population of this poor country.

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A novel coronavirus 2019-nCoV was first identified in the state of Wuhan, China, at the end of 2019. Coronaviruses are basically a group of viruses containing nonsegmented, single-stranded RNA. These groups of virusescaused several outbreaks around the world, like 'Severe Acute Respiratory Syndrome (SARS) pandemic'in 2002-2003 and the 'Middle East Respiratory Syndrome (MERS) outbreak' in South Korea in 2015. But most recently,COVID-19 triggered an outbreak in China in December 2019, subsequently become pandemic. Though some of corona patient reached in to devastating epidemics outcome, but others manifested mild to moderate respiratory infections, like the common cold.[1]

Ivermectin is a broad spectrum anti-parasiticagent[2] seems to have some anti-viral activities against a large number of viruses[3,4,5,6] in vitro.Originally identiûed as an inhibitor of inter action between the human immunodeûciency virus-1(HIV-1) integrase protein (IN) and the importin (IMP) á/â1 heterodimer responsible for IN nuclear import.[7] Studies on SARS-CoV proteins have

FOI 26-3132 - Document 5

revealed a potential role for IMPá/â1 during infection in signal dependent nucleocytoplasmic shutting of the SARS-CoVnucleo capsid protein,[8,9,10] that may impact host cell division,[11,12] addition of the SARS-CoV accessory protein ORF6 has been shown to antagonize the antiviral activity of the STAT1 transcription factor by sequestering IMPá/â1 on therough ER/ Golgi membrane13. Taken together, these reports suggested that Ivermectin's nuclear transport inhibitory activity may be eûective against SARS-CoV-2. When doxycycline was combined with macrocyclic lactonesin adult worms, ivermectin efficacy was 80% compared with 9% when treatment was performed with doxycycline alone 14 The largest dose intensity with registered pharmacokinetic parameters in healthy subjects is 120 mg, corresponding to up to 2000 µg/kg.[15]

HCQ has shown antiviral effects at both preinfection and postinfection stages. HCQ could interfere with the glycosylation of angiotensin-converting enzyme 2, thereby reducing the binding efficiency between angiotensin-converting enzyme 2 on host cells and the SARS-CoV-2 spike protein. Moreover, HCQ could be able to block virus fusion with the host cell through the inhibition of protease activity in cleaving corona virus surface spike proteins.[16]

Far less is known about the antiviral effects of azythromicin, which has been suggested to interfere with influenza virus internalization[17]. Interestingly, CLQ is also considered as an inhibitor of endocytic pathways through an elevation of endosomal pH[18]. azithromycin was added to hydroxychloroquine, the efficiency of SARS CoV-2 elimination was significantly improved.[19]

Regarding diagnosis till now no test is gold standard making it more challenging in diagnosing the disease.Specificity of RT-PCR is dependent on site, like 63% for nasal swabs, and only 32% for throat/ pharyngeal swabs.[20]

Method:

It's a prospective comparative studyconducted at Combined Military Hospital Dhaka. Total 400 Covid-19 PCR positive patients were included in this study. Among them 200 cases received ivermectin 18 mg on first day and Doxycycline 100 mg twice daily for 05 days comprising Group A and the rest 200 patients were given hydroxichloroquine 800 mg on first day then 400mg daily for 10 days and azithromycin 500 mg one day then 250 g daily for 4 days comprised Group B.Diagnosis of Covid-19 was done by RT-PCR from nasal and pharyngeal swab. The patients who had mild to moderate disease according to 'National Guideline on clinical management of coronavirus disease 2019 (COVID-19) version 7.0' were included in this study. Severe and critical patients were excluded from the study.Patientswere admitted in corona ward in April 2020 were given RT- PCR on 11th day and 12th day in Group B after completion of the recommended dose. Whereas the PCR test were done on 5th and 6th day after ingestion of Ivermectin in corona ward during May 2020 in Group A. The date of check Rt-PCR was determined by the recommended dose of different drugs used in this study.

Result:

Total 400 patients were included in this study. None developed severe or critical disease during study.

Table-I

Demographic profile (n=400)

Demographic profile	Group A	Group B
Mean age in years	36.4	39.42
Age range in years	17-74	20-79
Male	78.5%(157)	188(94%)
Female	21.5%(43)	12(6%)

Above table shows demographic profile in which 78.5% (157) were male; 21.5% (43) were Female in Group A and 188 (94%) male and 12 (6%) female in group B. Their age range was 17-74 with mean 36.46 years in group A and in group B age range 20-79 with mean 39.42 years.

Symptoms of the patients during admission (n=400)

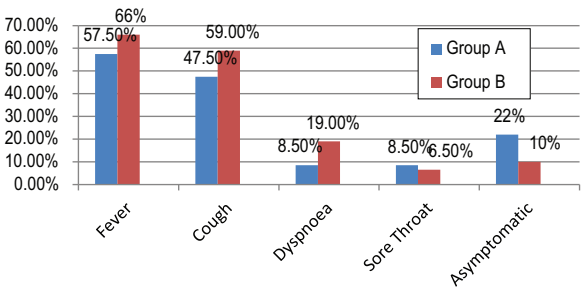


Fig.-1: *Showing Symptoms of Patient during admission*

FOI 26-3132 - Document 5

It shows that maximum patient had fever 57.5% and 66% in group A and B. Then next common symptom was Cough 47.5% and 59%. There were 22% patient who had no Covid symptoms in group A and only 10% in group B

Co-Morbidities of the patients (n=400)

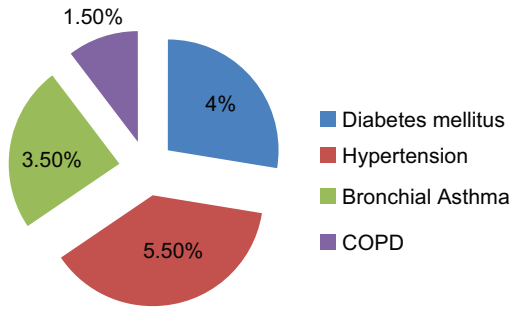


Fig.-2: *Pie chart Showing Co-morbidities of Group A*

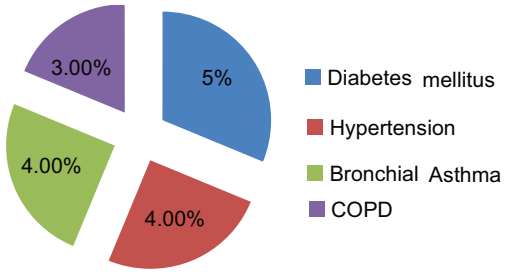


Fig.-3. *Pie chart showing Co- morbidities of GroupB*

Among the patients 11 (5.5%) had hypertension in group A and8 (4%) in Group B, 8(4%) and10 (5%) had diabetes mellitus respectively, 7(3.5 %) and 8(4%) had Bronchial Asthma and 3 (1.50 %)and 6 (3%) had COPD.

Viral Clearance of Group A and B (n=400)

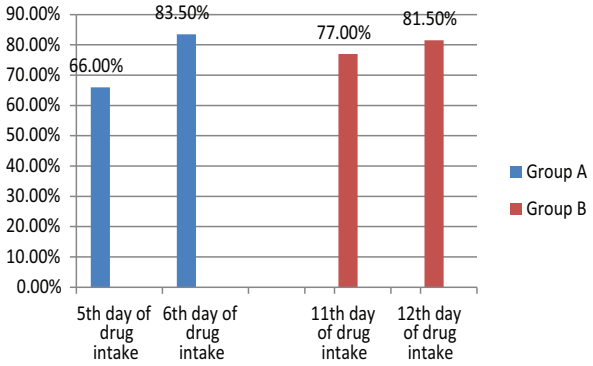


Fig.-4: *showing Viral Clearance*

Diagram showing viral clearance is 132 (66%) on day 5 and 167(83.5%) on day 6. Among them 33(16.5%) remain PCR positive after 6th day of ivermectin ingestion in Group A. Whereas there is154 (77.0%) viral clearance at 11th day and 163(81.5%) viral clearance at 12th day of hydroxychloroquine ingestion in Group B. Among them 37 (18.5%) remain PCR positive after 12 day in group B. The P value is 0.000427 which is significant considering 5th day viral clearance of ivermectin ingestion and 11th day of hydroxychloroquine ingestion. But considering 6th day and 12th day the P-value is 0.59 which is not significant.

Table-II

Uncommon Symptoms in Group A and B

Group A (n=200)		Group B (n=200)	
Symptoms	n (%)	Symptoms	n (%)
Anorexia	47 (23.5)	Anorexia	62 (31)
Vomiting	7 (3.5)	Vomiting	4 (2)
Diarrhoea	24 (12)	Diarrhoea	14 (7)
Skin Rash	20 (10)	Skin Rash	2 (01)

Above table shows some uncommon symptoms of the disease which can also be considered as drug effect due to sharing of the nature of presentation where anorexia (23.5% and 31 %) is the predominant feature is also a documented symptoms of Covid. Next effect was diarrhea (12%) in group A. Others were skin rash (10% and 1 %) and vomiting (3.5% and 2 %).

Table-III

Liver and Renal biochemical changes after drug intake in Group A and B

Group A	Group B	
Parameter	Mean	Mean
Serum Bilirubin	0.55±0.19 mg/dl	0.58±0.07 mg/dl
ALT	34.16±21.7 IU/l	29.16±18.8 IU/l
ALP	79.95±28.56 IU/l	88.6±23.77 IU/l
GGT	51.23±56.18 IU/l	49.23±66.18 IU/l
INR	1.142±0.084	1.1±0.062
Serum Creatinine	0.87±0.33 mg/dl	0.91±0.44 mg/dl

Above table showing the distribution of the liver and renal biochemical parameters in Group A and B. It shows that Bilirubinelevel is0.55 mg/dl and 0.58 mg/dl respectively. ALT level is 34.16 IU/L and 29.16 IU/L. ALP is 79.95 and 88.6 IU/Lin groupA and B. GGT is 51.23 IU/L and 49.23 IU/L. INR is 1.14 and 1.1 respectively.

FOI 26-3132 - Document 5

Creatinine is 0.87 mg/dl and 0.91 mg/dl in Group A and group B.

Discussion:

The search of effective anti-viral treatment against COVID-19 has led us to look into the existing drugs with anti-viral properties which may be effective against this novel CORONA virus. Ivermectin is a well-established anti-parasite drug used in both human and livestock animals. In-vitro studies have found profound anti-viral effect of this drug against several RNA viruses5including SARS-CoV-2.21 doxycycline has been is claimed to have antiviral properties by several mechanisms. 22It may also have a role of the anti-inflammatory effect of doxycycline in COVID-19 infection.23In this study, 200 patients received the combination of ivermectin (18 mg single dose) and Doxycycline (100 mg twice daily for 5 days). Majority (78.5%) were male, mean age was 36 years (range 17 to 74 years).Fever (57.5%) was the commonest symptom followed by cough (47.5%), sore throat (8.5%), dyspnoea (8.5%). A significant number of patients (22%) were asymptomatic (Non-specific symptoms) in Group-A. On 5thand 6thday viral clearance were achieved by 66% and 83.5% respectively. ivermectin and doxycycline combination were well tolerated by the patients though some unusual symptoms were seen might have been attributed to the effect of drugs or to rare Covid related symptoms which were anorexia (23.5%), diarrhoea (12%), skin rash (10%) and vomiting (3.5%). No significant renal or hepatic impairment was found. ivermectin was found to be safe at current dose. 24 In Group-B Fever (66%) was also the commonest symptom followed by cough (59%), sore throat (6.5%), dyspnoea (19%). Asymptomatic (Non-specific symptoms) patients were only 10%. The effect of hydroxychloroquine (HCQ) with or without azithromycin, on COVID-19 was promising in several studies where HCQ and azithromycin showed earlier viral clearance than placebo. 25 In this study, 200 patients received HCQ & azithromycin combination. Mean age was 39.4 years (range 20-79 years) and 94% were male. All had mild to moderate disease.Viral clearance by RT-PCR of nasal and pharyngeal swab were 77% at day 11 and 81.5% at day 12. No major adverse effect were observed other than minor overlapping symptoms (anorexia 31%, diarrhoea 7%, vomiting 2%, skin rash 1%). In this study only mild to moderate COVID-19 cases were included. ivermectin and doxycycline combination was associated with earlier viral clearance than HCQ & azithromycin combination. No severe adverse effect was observed in either group. This is a new study; So far no definite study was found on human being affecting viral clearance of covid-19. This is a observational study and we have choose very low dose of ivermectin keeping consideration of individual safety.The exact dose which will be absolutely beneficial for viral clearance of covid-19 on human being need to be determined in future by further control study.

Limitations

There are few limitations of the study like a) no severe cases were included in this study, b) effect of drug combination on clinical follow up outcome were not assessed and compared, c) false negative result could not be excluded and d) cardiac effect due to drugs could not be monitored. e) Effective dose of Ivermectin to clear virus not measured.

Conclusion:

Ivermectin and Doxycycline is a safe combination and found effective in early viral clearance in mild to moderate COVID-19 patients. On the other hand Hydroxychloroquine (HCQ) and Azithromycinewas also found safe but took more time in viral clearance and it itself might putting extra adverse effect on already compromised heart from viral effect. Nevertheles further control study is required to know more about the effects of ivermectin and doxycyclin on covid -19 patient

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Use of Ivermectin Is Associated With Lower Mortality in Hospitalized Patients With Coronavirus Disease 2019

The Ivermectin in COVID Nineteen Study

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BACKGROUND: Ivermectin was shown to inhibit severe acute respiratory syndrome coronavirus 2 replication in vitro, which has led to off-label use, but clinical efficacy has not been described previously.

RESEARCH QUESTION: Does ivermectin benefit hospitalized coronavirus disease 2019 (COVID-19) patients?

STUDY DESIGN AND METHODS: Charts of consecutive patients hospitalized at four Broward Health hospitals in Florida with confirmed COVID-19 between March 15 and May 11, 2020, treated with or without ivermectin were reviewed. Hospital ivermectin dosing guidelines were provided, but treatment decisions were at the treating physician's discretion. The primary outcome was all-cause in-hospital mortality. Secondary outcomes included mortality in patients with severe pulmonary involvement, extubation rates for mechanically ventilated patients, and length of stay. Severe pulmonary involvement was defined as need for $F_{IO_2} \geq 50\%$, noninvasive ventilation, or invasive ventilation at study entry. Logistic regression and propensity score matching were used to adjust for confounders.

RESULTS: Two hundred eighty patients, 173 treated with ivermectin and 107 without ivermectin, were reviewed. Most patients in both groups also received hydroxychloroquine, azithromycin, or both. Univariate analysis showed lower mortality in the ivermectin group (15.0% vs 25.2%; OR, 0.52; 95% CI, 0.29-0.96; $P = .03$). Mortality also was lower among ivermectin-treated patients with severe pulmonary involvement (38.8% vs 80.7%; OR, 0.15; 95% CI, 0.05-0.47; $P = .001$). No significant differences were found in extubation rates (36.1% vs 15.4%; OR, 3.11; 95% CI, 0.88-11.00; $P = .07$) or length of stay. After multivariate adjustment for confounders and mortality risks, the mortality difference remained significant (OR, 0.27; 95% CI, 0.09-0.80; $P = .03$). One hundred ninety-six patients were included in the propensity-matched cohort. Mortality was significantly lower in the ivermectin group (13.3% vs 24.5%; OR, 0.47; 95% CI, 0.22-0.99; $P < .05$), an 11.2% (95% CI, 0.38%-22.1%) absolute risk reduction, with a number needed to treat of 8.9 (95% CI, 4.5-263).

INTERPRETATION: Ivermectin treatment was associated with lower mortality during treatment of COVID-19, especially in patients with severe pulmonary involvement. Randomized controlled trials are needed to confirm these findings. CHEST 2021; 159(1):85-92

KEY WORDS: hospitalized COVID-19; in-hospital mortality; ivermectin; mechanical ventilation; number needed to treat; severe pulmonary involvement; survival

ABBREVIATIONS: COVID-19 = coronavirus disease 2019; IQR = interquartile range; MAP = mean arteria pressure; QTc = corrected QT interval; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

AFFILIATIONS: From the Broward Health Medical Center, Fort Lauderdale, FL (J. C. R., N. F., F. V., J. S., and J.-J. R.); the Drexel University College of Medicine (M. S. S.),

Take-home Points

Study Question: Is ivermectin associated with lower mortality rate in patients hospitalized with coronavirus disease 2019 (COVID-19)?

Results: A retrospective cohort study of consecutive patients hospitalized with confirmed severe acute respiratory syndrome coronavirus 2 infection at a four-hospital consortium in South Florida. Analysis showed statistically significant lower mortality rates in the group treated with ivermectin as compared with the group treated with usual care (15.0% vs 25.2%).

Interpretation: Ivermectin was associated with lower mortality during treatment of COVID-19 patients, especially in patients who required higher inspired oxygen or ventilatory support.

Ivermectin previously was studied as a therapeutic option for viral infections, with data showing some in vitro activity against a broad range of viruses, including HIV, dengue, influenza, and Zika virus, likely through inhibition of importin α/β1-mediated nuclear import of viral proteins.[1-3] Wagstaff et al[4] demonstrated that ivermectin was a potent in vitro inhibitor of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), showing a 99.8% reduction in viral RNA after 48 h. Reports can be found on the Internet of physicians worldwide treating Coronavirus disease 2019 (COVID-19) empirically with ivermectin since late April 2020. According to ClinicalTrials.gov, currently 37 studies are investigating the usefulness of ivermectin in COVID-19. However, in vivo efficacy of ivermectin in SARS-CoV-2 infection in humans has not been reported previously.

In the late 1970s, ivermectin was developed as a new class of drug to treat parasitic infections. Initially used in veterinary medicine, it soon was found to be safe and effective in humans. It has been used successfully to treat onchocerciasis and lymphatic filariasis in millions of people worldwide as part of a global drug donation program. About 3.7 billion doses of ivermectin have been distributed in mass drug administration campaigns globally over the past 30 years. Presently, ivermectin is approved for use in humans in several countries to treat onchocerciasis, lymphatic filariasis, strongyloidiasis, and scabies.[1]

Based on the data drug safety sheet for ivermectin (New Drug Application Identifier: 50-742/S-022), side effects were uncommon and limited. Reported side effects with more than 1% occurrence included elevation in alanine aminotransferase and aspartate aminotransferase (2%), nausea (2%), diarrhea (2%), decreased leukocyte count (3%), peripheral edema (3%), tachycardia (3%), dizziness (3%), and pruritus (3%). A pharmacokinetic study of 166 patients reported side effects of headache (6%), dysmenorrhea (5.5%), upper respiratory infection symptoms (1.8%), and diarrhea (1.8%).[5]

Methods

Patients

Sequentially consecutive hospitalized patients at four Broward Health-associated hospitals in South Florida with laboratory-confirmed infection with SARS-CoV-2 during their admission were reviewed in this study. The list of confirmed cases was provided by the hospitals' epidemiology departments. Enrollment dates ranged from March 15, 2020, through May 11, 2020. Confirmatory testing was performed by nasopharyngeal swab using a Food and Drug Administration Emergency Use Authorized COVID-19 molecular assay for the detection of SARS-CoV-2 RNA. Patients younger than 18 years and those who were pregnant or incarcerated were excluded from data collection based on institutional review board requirements. Patients who had at least two separate admissions placing them in both groups also were excluded.

Study Procedures

Records were abstracted by four of the authors (J. C. R., N. F., J. S., and J.-J. R.), and all data were reviewed subsequently and confirmed by the lead author. Baseline data were collected at the time of ivermectin administration for the ivermectin group; for the usual care group, baseline was either the time of administration of hydroxychloroquine or, if not used, at the time of admission. Information collected included COVID-19 testing results, patient demographics, pre-existing comorbid conditions, initial vital signs, laboratory results, and the use of corticosteroids, hydroxychloroquine, and azithromycin to describe the cohort and to identify potential confounders between groups. Severity of pulmonary involvement was assessed at the time of baseline data collection and was categorized

Philadelphia, PA; and the Florida International University (J.-J. R.), Miami, FL.

FUNDING/SUPPORT: The authors have reported to *CHEST* that no funding was received for this study.

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DOI: https://doi.org/10.1016/j.chest.2020.10.009

as severe or nonsevere. Patients were considered to have severe pulmonary involvement if they required an F_{IO_2} of 50% or more, high-flow nasal oxygen, noninvasive ventilation, or intubation and mechanical ventilation. The nonsevere pulmonary criteria encompassed patients who required no supplemental oxygen or low F_{IO_2} (ie, venturi mask 40% or less or up to 6 L/min of low-flow nasal cannula), independent of laboratory findings.

Patients were categorized into two treatment groups based on whether they received ivermectin at any time during the hospitalization. Patients in the ivermectin group received at least one oral dose of ivermectin at 200 μg/kg in addition to usual clinical care. A second dose could be given at the discretion of the treating physician at day 7 of treatment. Ivermectin is not currently approved by the Food and Drug Administration for COVID-19 treatment. The decision to prescribe ivermectin, hydroxychloroquine, azithromycin, or other medications was at the discretion of the treating physicians; however, hospital guidelines were established for the safe use and dosing of these agents. These guidelines included a baseline ECG and mandatory cardiac and corrected QT interval (QTc) monitoring for patients receiving hydroxychloroquine (alone or in combination with azithromycin), avoidance of azithromycin if patient's baseline QTc was more than 460 msec, and discontinuation of hydroxychloroquine if a concerning elevation in QTc occurred or if the patient's cardiologist recommended discontinuation. Oxygen and ventilatory support were applied per the customary care. Empiric use of ivermectin was given explicitly for COVID-19.

Outcomes

The primary outcome was all-cause in-hospital mortality. A patient was considered a survivor if he or she left the hospital alive or if his or her status in the hospital changed from active care to awaiting transfer to a skilled facility. Two consecutive nasopharyngeal swab specimens showing negative results for SARS-CoV-2, collected ≥ 24 h apart, were necessary for a patient to be accepted to the local skilled nursing facilities.

Secondary outcomes included subgroup mortality of patients with severe pulmonary involvement, extubation rates for patients requiring mechanical ventilation, and length of hospital stay. Length of stay was calculated from day of admission to either the day of discharge or to patient death.

Statistical Analysis

Univariate analysis of the primary mortality outcome and comparisons between treatment groups were determined by the Student *t* test for parametric continuous variables or the Mann-Whitney *U* test for nonparametric continuous variables as appropriate, and by the Pearson χ^2 test for categorical variables. The method of Hodges-Lehman was used to estimate median differences with 95% CIs.

To adjust for confounders and between-group differences, a multivariate analysis was performed using stepwise binary logistic regression. Patient variables included in the analysis were age, sex, comorbidities of diabetes, chronic lung disease, cardiovascular disease, and hypertension, smoking status, severity of pulmonary involvement, need for mechanical ventilation at study entry, BMI, peripheral white blood count, absolute lymphocyte count, and use of corticosteroids based on bivariate associations within our data, a priori plausibility, and documented associations with mortality from previous studies. Adjusted ORs with 95% CIs were computed to show level of certainty. Analyses were based on nonmissing data, and missing data were not imputed. Missingness of 1% was found for peripheral WBC count, 5% for smoking status, and 7% for absolute lymphocyte count.

We performed a secondary analysis using propensity score matching to reduce the effects of confounding and the likelihood of selection bias. Propensity matching was performed using a nearest-neighbor algorithm with 1:1 matching without replacement and a caliper distance of less than 0.2. Variables for propensity scoring included those variables from the univariate between-groups analysis of the unmatched cohort that had a *P* value of less than .2 (age, sex, pulmonary condition, hypertension , HIV status, severe pulmonary presentation, and exposure to corticosteroids, hydroxychloroquine, or azithromycin). Race, WBC count, absolute lymphocyte count, and need for mechanical ventilation before or on the day of study entry also were added as potential clinical confounders.

All tests were two-sided and a P value $< .05$ was considered statistically significant. Statistical analyses were conducted using IBM SPSS version 26.0 software, R version 3.5.3 software (R Foundation for Statistical Computing), and SPSS PS-matching software (sourceforge.net).

This study was conducted in accordance with tenets of the amended Declaration of Helsinki. The protocol was approved by the institutional review board for the Broward Health Hospital System (Identifier: 2020-034-BHMC). The authors assume responsibility for the accuracy and completeness of the data and analyses, as well as for the fidelity of the study.

Results

Characteristics of the Patients

Three hundred seven patients were admitted for COVID-19 during the period studied. Four patients were not reviewed because of multiple admissions, 11 did not have COVID-19 confirmed at the time of the study, and 12 were excluded because their age was younger than 18 years, they were pregnant, or they were incarcerated. The remaining cohort of 280 patients comprised 173 treated with ivermectin and 107 in the usual care group. Most patients received a single dose of ivermectin; however, 13 patients received a second dose of ivermectin for ongoing signs or symptoms on day 7 of treatment. Follow-up data for all outcomes were available through May 19, 2020. No patients were lost to follow-up for the primary outcome. At the time of analysis, all patients in both groups had met the end point of death, discharge alive, or awaiting transfer to a skilled facility. Of those awaiting transfer, in the control group, one patient was awaiting transfer to hospice because of an unrelated terminal illness and one patient was awaiting negative COVID-19 test results to proceed with unrelated surgery. In the ivermectin group, five patients were in stable condition, awaiting transfer to skilled facility or rehabilitation, and one patient was improving clinically.

Baseline characteristics and between-group comparisons for unmatched and propensity-matched cohorts are shown in Table 1. Before matching, hypertension and

FOI 26-3132 - Document 5

TABLE 1] Patient Characteristics by Treatment Group

Demographic Characteristic	Unmatched Cohort				Matched Cohort			
	Total (N = 280)	Usual Care (n = 107)	Ivermectin (n = 173)	*P* Value	Total (N = 196)	Usual Care (n = 98)	Ivermectin (n = 98)	*P* Value
Age, y	59.6 ± 17.9	58.6 ± 18.5	60.2 ± 17.6	.45	59.6 ± 17.5	59.04 ± 17.7	60.07 ± 17.4	.68
Female sex	127 (45.4)	43 (41.2)	84 (48.6)	.17	78 (39.8)	39 (39.8)	39 (39.8)	1.0
Race or ethnicity			.36				1.0	
Black	153 (54.6)	55 (51.4)	98 (56.6)		108 (55.1)	54 (55.1)	54 (55.1)	
White	76 (27.1)	35 (32.7)	41 (23.7)		55 (28.1)	27 (27.6)	28 (28.6)	
Hispanic	33 (11.7)	12 (11.2)	21 (12.1)		23 (11.7)	12 (12.5)	111 (11.2)	
Other or not identified[a]	13 (4.6)	5 (4.7)	13 (7.5)		10 (5.1)	5 (5.1)	5 (5.1)	
Current or former smoker	46/255 (18.0)	22/99 (22.3)	24/156 (15.6)	.40	31/180 (22.2)	20/90 (22.2)	11/90 (12.2)	.11
No. of comorbidities	1.66 ± 1.34	1.60 ± 1.46	1.70 ± 1.27	.57	1.56 ± 1.33	1.58 ± 1.43	1.53 ± 1.22	.79
Diabetes	90 ± 32.1	31 ± 29.0	59 ± 34.1	.37	59 ± 30.1	30 ± 30.6	29 ± 29.6	.88
Cardiac	43 ± 15.4	18 ± 16.8	25 ± 14.5	.59	27 ± 13.8	16 ± 16.3	11 ± 11.2	.30
Pulmonary	28 ± 10.0	14 ± 13.1	14 ± 8.9	.18	18 ± 10.1	10 ± 10.2	8 ± 8.2	.62
Obesity	114 ± 40.7	42 ± 39.3	72 ± 41.6	.70	79 ± 40.3	39 ± 39.8	40 ± 40.1	.88
Renal	24 ± 8.6	10 ± 9.4	14 ± 8.1	.72	16 ± 8.2	9 ± 9.2	7 ± 7.1	.60
Cancer	17 ± 6.1	8 ± 7.5	9 ± 5.2	.44	14 ± 7.1	7 ± 7.1	7 ± 7.1	1.00
Hypertension	50 ± 17.9	13 ± 12.2	37 ± 21.4	.05	26 ± 13.2	12 ± 12.2	14 ± 14.3	.67
Neurologic	28 ± 10.0	8 ± 7.5	20 ± 11.6	.27	17 ± 8.7	8 ± 8.2	9 ± 9.2	.80
HIV infection	9 ± 3.2	1 ± 1	8 ± 4.6	.09	3 ± 1.5	1 ± 1.0	2 ± 2.0	.56
Thyroid	23 ± 8.2	7 ± 6.6	16 ± 9.3	.42	15 ± 7.7	7 ± 7.1	8 ± 8.2	.79
BMI	30.0 ± 7.8	29.8 ± 7.2	30.1 ± 8.2	.81	29.4 ± 6.6	29.4 ± 6.3	29.4 ± 6.9	.95
Pulmonary severity				.46				
Severe	75 (26.8)	26 (24.3)	49 (28.3)	.12	47 (24.0)	22 (22.4)	25 (25.5)	.62
Intubated at study entry	38 (13.6)	15 (14.0)	23 (13.3)	.86	25 (12.8)	11 (11.2)	14 (14.3)	.52
Heart rate	86.0 (75.0-98.0)	86.0 (74.0-97.0)	86.0 (75.5-98.0)	.65	85.5 (74.0-98.0)	86.0 (73.0-97.5)	85.0 (74-98.0)	.88
MAP (mm Hg)	93 (82.3-103.0)	90 (81.0-103.0)	94 (83-103)	.24	92.5 (82.0-103.0)	91.0 (81.0-103.2)	93.0 (82.0-103.0)	.74
MAP ≤ 70 mm Hg	13/260 (5.0)	6/89 (6.7)	7/171 (4.1)	.35	7 (3.6)	4 (4.1)	3 (3.1)	.70
Corticosteroid	90 (32.1)	21 (19.6)	69 (39.8)	.001	46 (23.2)	21 (21.4)	25 (25.5)	.5
Hydroxychloroquine	260 (92.9)	104 (97.2)	156 (90.2)	.03	190 (96.9)	95 (96.9)	95 (96.9)	1.00
Azithromycin	243 (86.7)	99 (92.5)	144 (83.2)	.03	177 (90.3)	90 (91.8)	87 (88.7)	.47

(Continued)

TABLE 1 *(Continued)*

Demographic Characteristic	Unmatched Cohort				Matched Cohort			
	Total (N = 280)	Usual Care (n = 107)	Ivermectin (n = 173)	*P* Value	Total (N = 196)	Usual Care (n = 98)	Ivermectin (n = 98)	*P* Value
Peripheral WBC count ($\times 10^9$/L)	7.3 (5.6-10.2; n = 277)	7.0 (5.7-8.9; n = 106)	7.6 (5.5-11.1; n = 171)	.41	6.9 (5.3-9.3)	7.0 (5.8-9.0)	6.9 (5.2-9.8)	.69
Lymphocyte count ($\times 10^9$/L)	1.15 (0.78-1.56; n = 260)	1.14 (0.84-1.49; n = 102)	1.20 (0.77-1.67; n = 158)	.62	1.13 (0.77-1.52)	1.15 (0.87-1.45)	1.19 (0.75-1.57)	.88

Data are presented as No. (%), mean ± SD, or median (interquartile range), unless otherwise indicated. Current and former smoker is given as a proportion of the population with known smoking status documented in their medical records. MAP = mean arterial pressure.
[a]Asian, Native American, Pacific Islander, or not identified.

corticosteroid use were more prevalent in the ivermectin group, whereas the use of hydroxychloroquine and hydroxychloroquine plus azithromycin were higher in the usual care group.

Propensity score matching created a total of 98 matched pairs. After matching, no statistically significant differences were found between the two groups. Eight patients in the propensity-matched group received a second dose of ivermectin on day 7.

Outcomes

Unadjusted outcomes for the unmatched cohort and outcomes in the propensity-matched cohort are shown in Table 2. For the unmatched cohort, overall mortality was significantly lower in the ivermectin group than in the usual care group (15.0% vs 25.2% for ivermectin and usual care, respectively; $P = .03$). Mortality also was lower for ivermectin-treated patients in the subgroup of patients with severe pulmonary involvement (38.8% vs. 80.7% for ivermectin and usual care, respectively; $P = .001$). On univariate analysis, patients receiving corticosteroids showed a higher mortality than those who did not receive corticosteroids (30.0% vs 13.7%; OR, 2.7; 95% CI, 1.47-4.99; $P = .001$); however, corticosteroids were more likely to have been prescribed for severe patients (58.6% vs 22.4% for severe and nonsevere, respectively; OR, 4.91; 95% CI, 2.78-8.63; $P < .001$).

Results were similar, with lower mortality in the ivermectin-treated patients for the matched cohort for the group as a whole and for the subgroup with severe pulmonary involvement (Table 2). In the matched cohort, ivermectin was associated with an absolute risk reduction of 11.2% (95% CI, 0.38%-22.1%) and a corresponding number needed to treat of 8.9 (95% CI, 4.5-263) to prevent one death. We found no difference in median hospital length of stay or in extubation rates in either the unmatched or matched cohorts. Of note, 1 of the 13 patients who received a second dose of ivermectin died; this patient was not in the propensity-matched cohort.

Multivariate analysis was performed on the unmatched cohort, adjusting for demographic factors and between-group differences in mortality risks. Independent predictors of in-hospital mortality included treatment group, age, severe pulmonary disease category, and reduced lymphocyte count (Table 3). Because race was not a significant predictor after adjustment, a further analysis was performed that showed that White patients were significantly older than Black patients (mean age, 66.8 vs 59.1 y; mean difference, 7.7 y; 95% CI, 3.0-12.4 y;

TABLE 2] Univariate Clinical Outcomes by Treatment Group

Outcome	Unmatched Cohort				Matched Cohort			
	Control Subjects (n = 107)	Ivermectin (n = 173)	OR or Difference (95% CI)	*P* Value	Control Subjects (n = 98)	Ivermectin (n = 98)	OR or Difference (95% CI)	*P* Value
Mortality	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Total	27 (25.2)	26 (15.0)	0.52 (0.29-0.96)	0.03	24 (24.5)	13 (13.3)	0.47 (0.22-0.99)	0.045
Severe	21/26 (80.7)	19/49 (38.8)	0.15 (0.05-0.47)	0.001	18/22 (81.8)	8/25 (32.0)	0.27 (0.08-0.92)	0.002
Nonsevere	6/81 (7.4)	7/124 (5.6)	0.75 (0.24-2.3)	0.61	6/76(7.9)	4/74 (5.4)	0.97 (0.61-1.54)	0.78
Successful extubation	4/26 (15.4)	13/36 (36.1)	3.11 (0.88-11.00)	0.07	3/22 (15.4)	7/18 (38.9)	1.91 (0.43-8.46)	0.14
Length of stay	7.0 (4.0-10.0)	7.0 (4.0-13.3)	0 (-1 to 2)	0.34	7.0 (4.0-10.0)	7.0 (3.0-13.0)	0 (-2 to 1)	0.88

Data are presented as No./Total No. (%) or median (interquartile range) unless otherwise indicated.

$P = .001$) and that Hispanic patients (mean age, 49.8 y; mean difference, 17.0 y; 95% CI, 9.6-24.4 y; $P < .001$).

Discussion

In this multihospital retrospective cohort study, we observed a significant association of ivermectin with improved survival for patients admitted with COVID-19. This association also was seen in the subset of patients with severe pulmonary disease. These findings were confirmed after multivariate adjustment for comorbidities and differences between groups, and also in a propensity score-matched cohort. Similar to other studies, we noted that older age, cardiac disease, current or former smoking, more severe pulmonary involvement at presentation, higher WBC counts, and lower lymphocyte counts emerged as risk markers for in-hospital mortality.

The overall mortality, and mortality in intubated patients, in our usual care group was similar to what was reported in previous studies. Richardson et al[6] reported an overall mortality of 21% in a New York City cohort, with a mortality of 88% in intubated patients. Zhou et al[7] reported 28.2% mortality in a cohort of hospitalized patients in Wuhan, China; the intubated patients showed a mortality of 96.9%. In contrast to Magagnoli et al,[8] we did not see a higher mortality effect for hydroxychloroquine. This may have been because of the small number of patients who were not treated with this agent; thus, our study was underpowered to detect a difference in mortality from hydroxychloroquine treatment. We also hypothesize that precautionary measures in the hospitals' protocol for hydroxychloroquine use could have prevented fatal arrhythmias from developing. These included baseline electrocardiography and daily QTc monitoring by telemetry for any patient receiving hydroxychloroquine or combination therapy, avoidance of azithromycin if patient's baseline QTc was more than 460 msec, and discontinuation of hydroxychloroquine if a concerning elevation in QTc occurred or if the patient's cardiologist recommended discontinuation. In contrast to Horby et al,[9] we did not find a mortality benefit for patients who were prescribed corticosteroids in our multivariate analysis, which included several severity covariates. These findings are likely explainable by physicians' choice to reserve use of corticosteroids for the most seriously ill patients, because the study was performed before the results of the Randomised Evaluation of COVID-19 Therapy (RECOVERY) trial were published.[9]

TABLE 3] Multivariate Analysis of Factors Associated With Mortality

Variable	OR (95% CI)	*P* Value
Treatment group	. . .	. . .
Ivermectin	0.27 (0.09-0.80)	.03
Control subject	Reference	. . .
Age	1.05 (1.02-1.09)	.003
Sex	. . .	. . .
Female	0.42 (0.24-1.82)	.42
Male	Reference	. . .
Smoking status	. . .	. . .
Current or former smoker	3.49 (0.71-17.32)	.13
Nonsmoker	Reference	. . .
Race	. . .	.18
Black	0.64 (0.21-1.94)	.43
Hispanic	0.14 (0.02-1.22)	.08
Other	0.62 (0.05-7.92)	.71
White	Reference	. . .
Comorbidities	. . .	. . .
Diabetes	1.17 (0.39-3.55)	.78
Cardiac	1.51 (0.43-5.22)	.52
Pulmonary	0.15 (0.20-1.84)	.15
Hypertension	0.72 (0.17-3.08)	.66
No comorbidities	Reference	. . .
BMI	0.97 (0.89-1.07)	.58
Severe presentation	11.41 (3.42-38.09)	< .001
Intubated at study entry	2.96 (0.73-12.06)	.13
MAP ≤ 70 mm Hg	1.82 (0.17-19.1)	.62
Corticosteroid treatment	1.71 (0.57-5.16)	.34
Peripheral WBC count	1.08 (0.96-1.23)	.22
Lymphocyte count	3.65 (1.25-10.60)	.02

MAP = mean arterial pressure.

We also did not confirm a higher risk of mortality in Black patients in comparison with White patients after controlling for age. Prior reports showed lower survival rates among Black and Hispanic patients[10]; however, Price et al[11] also found no racial differences in mortality. In our hospital population, White patients were significantly older, which is reflective of our catchment area and may be responsible for the discrepancy.

We did not observe a significant difference in hospital length of stay between the groups (median, 7 days for both groups) despite the lower mortality. Possible explanation could include delay in discharging patients to other facilities (skilled nursing facilities, inpatient rehabs, and so forth) because of a delay in obtaining required repeat COVID-19 testing results. Patients who died were included in length-of-stay measurements.

Use of mechanical ventilation was not adopted as an outcome of interest, because guidelines and practice patterns for intubation criteria changed throughout the length of the study. We were unable to determine ICU length of stay and ventilatory-free days in the ICU because overflow conditions during the pandemic placed critically ill patients in the emergency room and other non-ICU environments, and therefore, we could not determine ICU stay accurately. We did not find a lower mortality in the subgroup of nonsevere patients treated with ivermectin; however, our study was not powered to assess these differences because the overall mortality in nonsevere patients was low. Similarly, the study was not powered to determine whether extubation rates were higher in the ivermectin group. These should be investigated further with a larger randomized controlled trial.

Interpretation

Our study has several limitations. Because of the retrospective observational nature of the study, despite adjustment for known confounders and propensity score matching, we cannot exclude the possibility of unmeasured confounding factors. Although more of the control group was enrolled in the first weeks of the study, suggesting the possibility of timing bias, this may be offset by preferential treatment of more severe patients with ivermectin early in the study because of low initial availability. We also did not find consistently different mortality outcomes with time over the short duration of this study. We also did not find evidence of immortal time bias, because only one of the control patients died fewer than 5 days from admission, the average time from admission to death was 11 days, and the vast majority of patients received ivermectin in 2 days or fewer. If we omit the patient with potential immortal time from the analysis, the mortality difference remains significant in both unmatched (15.0% vs 24.5% for ivermectin and usual care, respectively; $P < .05$) and matched (12.4% vs 25.0% for ivermectin and usual care, respectively; $P < .03$) cohorts. Most of the studied patients received hydroxychloroquine with or without azithromycin, and we are unable to determine whether these medications had an added benefit or whether mortality would have been better in both groups without these agents.

We showed that ivermectin administration was associated significantly with lower mortality among patients with COVID-19, particularly in patients with more severe pulmonary involvement. Interpretation of these findings are tempered by the limitations of the retrospective design and the possibility of confounding. Appropriate dosing for this indication is not known, nor are the effects of ivermectin on viral load or in patients with milder disease. Further studies in appropriately designed randomized trials are recommended before any conclusions can be made.

Acknowledgments

Author contributions: J. C. R., lead author, had full access to all of the data in the study and contributed to the study design, data collection and interpretation, and writing of manuscript. M. S. S. provided data analysis and interpretation and contributed to writing of the manuscript. N. F. contributed to data collection and literature search. F. V. contributed to the study design and data collection. J. S. contributed to data collection and data organization. J.-J. R., corresponding author, contributed substantially to the study design, data analysis and interpretation, and the writing of the manuscript.

Financial/nonfinancial disclosures: None declared.

Other contributions: The authors thank Edward Gracely, PhD, for his support and advice with the statistical analysis, and William Rifkin, MD, for reviewing the manuscript.

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FOI 26-3132 - Document 5

Research Square

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A Computational Study of Ivermectin and Doxycycline Combination Drug Against SARS-CoV-2 Infection

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Research Article

Keywords: COVID-19, SARS-CoV-2, Ivermectin, Doxycycline, 3CLpro

DOI: https://doi.org/10.21203/rs.3.rs-755838/v1

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FOI 26-3132 - Document 5

Priority Updates from the Research Literature from the Family Physicians Inquiries Network

PURLs®

Zinc for the common cold—not *if*, but when

A new meta-analysis shows that zinc supplementation can reduce the duration and severity of a cold, if it's started early on.

Goutham Rao, MD;
Kate Rowland, MD
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The University of Chicago

PURLs EDITOR
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Cleveland Clinic

PRACTICE CHANGER

Advise patients to start taking zinc supplements (available as tablets, syrup, or lozenges) within 24 hours of the onset of a cold.[1]

Singh M, Das RR. Zinc for the common cold. *Cochrane Database Syst Rev.* 2011;(2):CD001364.

STRENGTH OF RECOMMENDATION

A: Based on a meta-analysis of 13 randomized placebo-controlled trials.

ILLUSTRATIVE CASE

A healthy 31-year-old woman schedules a visit because of congestion and rhinorrhea. She tells you that her cold symptoms developed in the last day or 2 and asks for something to speed her recovery. What can you suggest?

Americans experience roughly 500 million episodes of noninfluenza-related viral respiratory tract infections annually (ie, common colds), or roughly 2.5 episodes per person each year. The total economic burden of the common cold is nearly $40 billion annually.[2]

Most cold remedies offer little relief

We've all cared for patients with cold symptoms. And despite the desire to help, many of us have been frustrated by the fact that there is no cure for the common cold, and most over-the-counter therapies offer little or no relief.

What about zinc? Zinc has been in and out of favor as a treatment for the common cold, based on contradictory findings, since the first randomized trial was published in 1984.[3]

A 1998 systematic review (which did not include a quantitative synthesis of data) suggested that zinc was beneficial in reducing the duration and severity of cold symptoms.[4] A meta-analysis published in 2000 found that zinc was ineffective compared with placebo in reducing the likelihood that cold symptoms were present after 7 days.[5] A meta-analysis published in June 2011 concluded that zinc lozenges reduce the duration of cold symptoms by 12% to 48%, but only at daily doses >75 mg.[6]

These conflicting results have made it difficult to recommend zinc to patients—until now.

STUDY SUMMARY

Zinc for colds? Cochrane review provides convincing evidence

The 2011 Cochrane review that's the focus of this PURL[1] addressed the question: Does zinc reduce the duration and severity of the common cold? The review also assessed zinc's ability to prevent colds, although only 2 studies focused on prevention.

The researchers included only randomized, double-blind, placebo-controlled trials in which oral zinc supplementation was used for ≥5 consecutive days for treatment or ≥5 months for prevention. Studies included patients of any age, taking any dosage and any formulation of zinc.

Primary outcomes included the duration of symptoms, the severity of symptoms, and the incidence of the common cold in preven-

FOI 26-3132 - Document 5

tion studies. Secondary outcomes included the proportion of patients symptomatic after 3, 5, and 7 days of treatment, the time to resolution of individual symptoms (eg, cough), change in individual symptom scores, school absences, antibiotic use, and adverse effects.

Thirteen therapeutic trials and 2 prevention trials met the inclusion criteria. These studies, all of which were judged to be of high quality with a low risk of bias, had a total of 996 patients in the therapeutic trials and 394 patients in the prevention trials. Participants ranged in age from 1 to 65 years.

Therapeutic trials. In general, therapeutic studies included lozenges containing 10 to 24 mg zinc gluconate, with one lozenge taken every 1 to 4 hours during the day for 3 to 7 days. In one therapeutic trial that enrolled only children ages 1 to 10, the intervention group took zinc sulfate syrup (15 mg bid) for 10 days. In 11 of the 13 therapeutic studies, treatment began in the first 24 hours of symptoms; in the remaining 2, it began within 48 hours of symptoms.

Ten studies reported on duration of symptoms; 6 of them were similar enough to allow for pooling of results. The pooled results showed that patients who took zinc had a shorter duration of cold symptoms (0.97 days; 95% confidence interval [CI], -1.56 to -0.38), compared with those on placebo. Pooled results from 5 trials revealed that zinc significantly reduced the severity of symptoms by a standard effect size of 0.39 (95% CI, -0.77 to -0.02), which is considered a small to moderate effect.

Prevention trials. In one prevention trial, which included only children ages 6½ to 10 years, those in the intervention group took one 10-mg zinc sulfate tablet 6 days a week for 5 months. In the other trial, children in the intervention group took 15 mg zinc sulfate syrup daily for 7 months.

Pooled results from these 2 studies revealed that daily zinc supplementation substantially reduced the incidence of colds. The incident rate ratio (the number of children who developed colds while taking zinc compared with the number who developed colds while on placebo) was 0.64 (95% CI, 0.47-0.88). In the original trials, one study found a difference of 0.5 colds (1.7 in the control group vs 1.2 in the intervention group) per season, and the other found a difference of 1.8 colds per season (3.15 in the control group vs 1.37 in the intervention group).

WHAT'S NEW

Evidence of zinc's cold relief properties is conclusive

This Cochrane review provides convincing evidence from 13 randomized placebo-controlled trials that taking zinc soon after the onset of symptoms of the common cold significantly reduces both the duration and severity of symptoms. Zinc supplements are widely available over the counter, so you can recommend that patients take zinc the next time they develop a cold.

In addition, 2 prevention trials found that zinc can reduce the incidence of colds in children, whether it is taken as a syrup or in tablet form. There have been few trials of zinc for prophylaxis of the common cold, and no previous meta-analyses included preventive studies.[7] This Cochrane review substantiates the effectiveness of zinc for prophylaxis of the common cold in young children.

However, children need to take zinc daily for prophylaxis, which may be inconvenient. Long-term safety information is not yet available. Given these considerations, parents may choose to wait for additional evidence about safety before considering daily prophylaxis.

CAVEATS

Adverse effects, long-term use may create problems

In this meta-analysis, side effects from zinc were common. The 2 most frequently reported were bad taste (pooled odds ratio [OR], 2.64; 95% CI, 1.91-3.64) and nausea (pooled OR, 2.15; 95% CI, 1.44-3.23). When you recommend zinc, warn your patients about these adverse effects. The side effects are not severe, so patients can decide for themselves whether the benefit of a reduction in cold duration is worth the downside of nausea and a bad taste in the mouth.

It is also important to note that the trials included in the meta-analysis enrolled healthy children and adults ≤65 years old.

INSTANT POLL QUESTION

Do you recommend that patients take zinc to reduce the duration and severity of cold symptoms?

- ☐ *Yes, frequently*
- ☐ *No, but I plan to do so*
- ☐ *No; the side effects are unpleasant*
- ☐ *Other*_____

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Whether zinc benefits people with chronic illnesses (eg, chronic obstructive pulmonary disease) who develop colds is unknown.

Prolonged elevated serum zinc levels can interfere with copper metabolism, and the adverse effects of long-term use of zinc as prophylaxis are unknown. The trials included in the meta-analysis took place in relatively affluent countries in which zinc deficiency is uncommon. It is not known what impact zinc supplementation would have on people in poor countries.

Of the 15 studies included in the meta-analysis, 10 received support from pharmaceutical companies, 4 received support from foundations, and one received support from both.

CHALLENGES TO IMPLEMENTATION

When to talk to patients about zinc

Most patients do not seek medical care for colds. Those who do typically present only after having symptoms for several days, and it is not clear whether zinc supplementation has the same beneficial effects when started after the first 24 hours.[2]

Thus, you may have few opportunities in the office to recommend zinc for patients with colds, for whom there is evidence of immediate benefit. More likely, you'll need to incorporate a zinc recommendation into your overall advice about colds.

Zinc is available over the counter in various forms and dosage. After recommending zinc, you may be confronted with the question of which dose, brand, and formulation is best—a question which, unfortunately, remains unanswered. JFP

ACKNOWLEDGEMENT
The PURLs Surveillance System is supported in part by Grant Number UL1RR024999 from the National Center For Research Resources, a Clinical Translational Science Award to the University of Chicago. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

Zinc supplements are widely available over the counter, so you can recommend that patients take zinc the next time they develop a cold.

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This supplement was submitted by the Primary Care Education Consortium and supported by an educational grant from Takeda Pharmaceuticals North America, Inc.

FOI 26-3132 - Document 5

REVIEW

The Role of Zinc in Antiviral Immunity

Scott A Read,[1,2] Stephanie Obeid,[3] Chantelle Ahlenstiel,[3] and Golo Ahlenstiel[1,2]

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ABSTRACT

Zinc is an essential trace element that is crucial for growth, development, and the maintenance of immune function. Its influence reaches all organs and cell types, representing an integral component of approximately 10% of the human proteome, and encompassing hundreds of key enzymes and transcription factors. Zinc deficiency is strikingly common, affecting up to a quarter of the population in developing countries, but also affecting distinct populations in the developed world as a result of lifestyle, age, and disease-mediated factors. Consequently, zinc status is a critical factor that can influence antiviral immunity, particularly as zinc-deficient populations are often most at risk of acquiring viral infections such as HIV or hepatitis C virus. This review summarizes current basic science and clinical evidence examining zinc as a direct antiviral, as well as a stimulant of antiviral immunity. An abundance of evidence has accumulated over the past 50 y to demonstrate the antiviral activity of zinc against a variety of viruses, and via numerous mechanisms. The therapeutic use of zinc for viral infections such as herpes simplex virus and the common cold has stemmed from these findings; however, there remains much to be learned regarding the antiviral mechanisms and clinical benefit of zinc supplementation as a preventative and therapeutic treatment for viral infections. *Adv Nutr* 2019;10:696–710.

Keywords: zinc, virus, metallothionein, antiviral, immunity, zinc deficiency, zinc supplementation

Introduction

Zinc deficiency was first recognized by Prasad et al. >50 y ago in a malnourished group of individuals presenting with hepatosplenomegaly, dwarfism, hypogonadism, and an elevated risk of infection (1). Unbeknownst to Dr. Prasad and his colleagues at the time, their discovery would highlight the importance of zinc as an integral component of human physiology, and inspire decades of zinc research. It is now understood that zinc is the second-most abundant trace metal in the human body after iron, and an essential component of protein structure and function. Importantly, zinc is a structural constituent of ∼750 zinc-finger transcription factors (2) enabling gene transcription, and is a catalytic component of approximately 2000 enzymes, encompassing all 6 classes (hydrolase, transferase, oxido-reductase, ligase, lyase, and isomerase) (3). Hence, zinc is biologically essential for cellular processes, including growth and development, as well as DNA synthesis and RNA transcription (4).

The global prevalence of zinc deficiency is estimated to range from ∼17% to 20% (5, 6), with the vast majority occurring in developing countries of Africa and Asia. Although significantly less common in high-income nations, zinc deficiency occurs most frequently in the elderly, vegans/vegetarians, and individuals with chronic disease such as liver cirrhosis (7) or inflammatory bowel disease (8). Importantly, zinc deficiency results in a compromised immune system, as evidenced by thymic atrophy, lymphopenia, and defective lymphocyte responses in animal studies (9). These data underscore the importance of zinc nutrition, particularly in underdeveloped countries where the risk of infection is heightened because of poor sanitation, public health, and vaccination strategies (5).

This review focuses on the role of zinc as an essential micronutrient that is required to mount an effective antiviral response. Although zinc possesses direct antiviral properties (e.g. influenza), it is also critical in generating both innate and acquired (humoral) antiviral responses. To complicate matters, zinc is an integral component of many viral enzymes, proteases, and polymerases, highlighting the importance of regulating cellular and systemic zinc distribution to inhibit viral replication and dissemination.

Supported by Sylvia and Charles Viertel Charitable Foundation Investigatorship (VTL2015C022). Author disclosures: SAR, SO, CA, and GA, no conflicts of interest.
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Abbreviations used: EV, *epidermodysplasia verruciformis*; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HPV, human papilloma virus; HSV, herpes simplex virus; IRF, IFN regulatory factor; ISG, interferon stimulated gene; MT, metallothionein isoforms; MTF1, metal-responsive transcription factor; PRR, pattern recognition receptor; RdRp, RNA-dependent RNA polymerase; RT, reverse transcriptase; SOCS, suppressors of cytokine signaling; TLR, Toll-like receptor; ZIP, Zrt- and Irt-like proteins.

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Current Status of Knowledge

Zinc homeostasis and viral infection

Systemic and intracellular zinc are tightly regulated, such that free zinc ions (Zn^{2+}) represent a minimal fraction of total cellular zinc (~0.0001%) (10–12). The vast majority of zinc remains bound to zinc-binding proteins such as serum albumin or intracellular metallothionein proteins, where it can be transferred to zinc-binding enzymes and transcription factors as necessary. Zinc transport is principally mediated by 2 groups of proteins: the ZnT [solute-linked carrier 30 (*SLC30A*)] family, which is responsible for efflux of zinc outside the cell or influx into organelles, and the ZIP [Zrt- and Irt-like proteins (*SLC39A*)] family of proteins, which performs the opposite role, transporting zinc into the cytoplasm from extracellular sources or cellular organelles (13). The >30 human proteins responsible for zinc homeostasis collectively ensure that zinc does not become toxic in the case of dietary excess, nor limited in the case of dietary insufficiency. Of course, this balance cannot be maintained indefinitely, and may result in zinc-induced copper deficiency if consumed in excess (14), and severe zinc deficiency if it is lacking in the diet (1).

Sequestration and toxic accumulation of metals are well-documented antibacterial immune responses. Calprotectin is a prime example, binding and sequestering extracellular calcium and zinc, thus preventing bacterial and fungal overgrowth (15). Conversely, toxic endosomal zinc accumulation can inhibit intracellular *Mycobacterium* growth in macrophages (16). Unfortunately, these mechanisms are not well described in the case of viral infections, perhaps because of a lack of efficacy. Calprotectin, for example, has no proven antiviral role, nor is it significantly upregulated in response to viral gastroenteritis (17). This absence of a zinc-mediated antiviral response may reflect the "parasitic" nature of viral infection, hijacking host machinery to self-replicate. Changes in intracellular zinc concentrations necessary to inhibit viral replication may also prove toxic to eukaryotic cells for the same reason.

Although antiviral modulation of zinc homeostasis in humans remains unproven, papilloma viruses have evolved mechanisms to alter zinc homeostasis to favor viral replication and persistence (18). The human papilloma virus (HPV) E5 protein can interact with the zinc transporter ZnT-1 in complex with EVER2, thus stimulating nuclear accumulation of zinc (19). The ZnT-1:EVER2 complex responsible for zinc export from the nucleus is inhibited by HPV E5, subsequently increasing both nuclear zinc and the activation of AP1 (20), a transcription factor required for HPV genome expression. Interestingly, homozygous mutations in either EVER1 or EVER2 result in a rare condition termed *epidermodysplasia verruciformis* (EV). EV patients are particularly susceptible to HPV strains 5 and 8, which significantly increases the risk of developing nonmelanoma skin cancers. HPV strains 5 and 8 lack expression of the E5 protein, which may explain *1*) their limited replication in the normal population because of their inability to control zinc homeostasis, and *2*) the susceptibility of EV patients to strains 5 and 8 from the loss of EVER protein function, favoring HPV replication. Interestingly, HPV E5 genes have co-evolved with the major HPV oncogenes, E6 and E7, and indicate the potential involvement of E5 in carcinogenesis (21, 22). Clinical trials using both oral and topical zinc have proven effective for the treatment of viral warts, and will be reviewed in a later section.

Metallothioneins, zinc homeostasis, and antiviral activity. Metallothioneins are small, cysteine-rich proteins capable of binding divalent cations such as zinc and copper. As vessels for much of the labile intracellular zinc pool, metallothioneins possess numerous functions through their ability to bind and release metals from their thiol groups. These include storage and transfer of zinc ions and heavy metal detoxification, as well as involvement in oxidative stress, apoptosis, and immune responses (23). Humans express 4 metallothionein isoforms (MT1–4), including the ubiquitously expressed MT1 and MT2 genes (MT1A, B, E, F, G, H, I, J, L, M, X, MT2A), as well as MT3 and MT4 whose expression is limited, and function remains poorly understood (24). Importantly, MT1 and 2 gene expression is extremely responsive to zinc, and therefore serves as an ideal indicator of an individual's zinc status (25). Upon taking a zinc supplement, for example, an increase in protein-bound zinc in the bloodstream is internalized by cells in various tissues and organs through the ZIP transporters. In response to increased intracellular zinc, the metal-responsive transcription factor (MTF1) becomes active, and binds the metal responsive element in metallothionein gene promoters to upregulate their transcription (26). Although there are additional stimuli that influence metallothionein expression, this primarily occurs in a zinc-dependent fashion. Oxidative stress, for example, induces zinc release from metallothioneins as a mechanism to reduce reactive oxygen species generated by mitochondrial dysfunction or viral infection (26). Zinc released from metallothioneins binds MTF1 to stimulate additional metallothionein expression.

It should be noted that metallothioneins, although highly responsive to zinc, have long been classified as interferon stimulated genes (ISGs) (27). IFNs are immunostimulatory cytokines secreted from infected cells and nearby immune cells that induce the expression of hundreds of antiviral genes. They possess diverse roles including chemoattraction, immune cell activation, and direct antiviral activity. In response to IFNs, we suggest that there are 2 mechanisms of metallothionein induction. Most ISGs possess binding sites for STAT- or IFN regulatory factor (IRF) transcription factor-mediated expression, as is the case for MT1X and MT2A (28, 29). Other metallothioneins such as MT1F and MT1G do not possess known IFN regulatory regions in their promoters, but are instead more sensitive to zinc (28). IFNs stimulate an influx of zinc into the target cell, as is the case with some inflammatory cytokines such as IL-6, which in turn drives metallothionein expression.

Because metallothioneins possess such a diverse functional repertoire, their specific roles during viral infection remain undefined. However, both in vitro and in vivo studies have made it abundantly clear that metallothioneins are induced by viruses. The mechanisms often remain undefined; however, metallothionein expression has been attributed to zinc influx or redistribution (19, 28), by viral means, cytokine exposure, or oxidative stress (30). Metallothionein upregulation has been observed in response to measles virus (31), influenza (31, 32), HIV (33), hepatitis C virus (HCV) (34), and coxsackie virus (35), among others. In the case of HIV, zinc appears to be the key driver of metallothionein expression to favor viral persistence. HIV-infected monocytes demonstrate a significant increase in both MT1 gene expression as well as intracellular zinc (33). Elevated intracellular zinc increases monocyte resistance to apoptosis via inhibition of caspase 3 activation [as has been reported previously (36)], thus providing a reservoir for HIV replication. The role of metallothioneins remains unclear in this study; however, they have been described as negative regulators of apoptosis, albeit not through direct caspase 3 inhibition (37). Zinc and metallothioneins also facilitate human cytomegalovirus (HCMV) replication by activating the immediate-early HCMV promoter (38, 39). Kanekiyo et al. demonstrated that both zinc and metallothionein overexpression increased NF-κB binding in the HCMV promoter. Because no complex was detected between metallothionein and NF-κB, it was suggested that metallothioneins served as a zinc donor necessary for NF-κB binding. In addition, as NF-κB transcription factors are known potent activators of HIV and HSV replication, and several other viruses (40), metallothioneins may be proviral. Zinc has also been reported to inhibit NF-κB in numerous studies (41–43). Despite these contrasting data, Kim et al. have bridged these inconsistencies, demonstrating that MT2A can serve as a sink for excess zinc (44), thus limiting its proximity to NF-κB and favoring NF-κB-mediated transcription.

In the case of HCV infection, metallothioneins possess an antiviral role. Using a pan-metallothionein siRNA to knockdown all MT1 and 2 genes, we demonstrated both an increase in HCV replication and a decrease in intracellular zinc content in vitro (34). Interestingly, although $ZnSO_4$ can reduce HCV replication, this effect was ablated when metallothionein genes were knocked down. These data suggest that metallothioneins are either *1*) directly antiviral, potentially by sequestering zinc away from viral metalloproteins such as HCV NS5A (45), or *2*) indirectly antiviral by acting as zinc chaperones and facilitating antiviral signaling. Further, metallothioneins possess antiviral properties against other viruses as well, as demonstrated in an antiviral screen of 380 human ISGs performed by Schoggins et al. (46). Overexpression of multiple members of the MT1 family inhibited replication of flaviviruses including yellow fever virus and HCV, as well as the alphavirus Venezuelan equine encephalitis virus. This effect was not observed in West Nile virus, and Chikungunya virus. These data indicate that metallothioneins, like many ISGs, are selectively antiviral, perhaps reflecting specific viral zinc requirements during replication. This is particularly evident for HIV, which demonstrated an increase in viral replication as a result of metallothionein overexpression in the Schoggins et al. ISG screen (46), validating previous works (33).

Zinc as an antiviral: bench to bedside and back again

Many studies have evaluated the efficacy of zinc as an antiviral agent in vitro. Unfortunately, zinc concentrations used to assess antiviral activity often far exceed physiological concentrations. Human plasma zinc, for example, ranges from approximately 10 to 18 μM (47), whereas antiviral concentrations of zinc can reach into mM concentrations (48). Intracellular zinc concentrations range from 10s to 100s of μM, but are significantly buffered by zinc-binding proteins such as metallothioneins, rendering free zinc concentrations at picomolar to low nanomolar concentrations (49, 50). The antiviral properties of zinc are certainly virus-specific, but it would appear that zinc ion availability plays a significant role in the antiviral efficacy of zinc (51). Here we describe the role of zinc as a virus-specific antiviral: both in vitro mechanistic studies, as well as human-based clinical trials using zinc supplementation. In vitro and in vivo studies are summarized in **Tables 1** and **2**, respectively.

Herpesviridae.

The effect of zinc on HSV-1 and -2 has been studied for >40 y, with in vitro studies suggesting that zinc plays an inhibitory role on almost every aspect of the viral life cycle: viral polymerase function (52), protein production and processing (53), and free virus inactivation (48, 54). Although these studies were performed >20 y ago, a more recent study using the zinc ionophore pyrithione demonstrated a reduction in HSV replication from reduced NF-κB activation by interfering with the protein ubiquitination pathway (41). Unfortunately, no recent experimental data can demonstrate with any certainty the mechanism by which zinc inhibits HSV infection. Nonetheless, in vivo studies in mice and humans have shown a significant reduction of infection and disease burden. Mouse studies performing intravaginal zinc inoculation in liquid (55) or gel (56) form both resulted in significant reductions in HSV-2 infection. Several topical zinc application studies have been performed in humans, which demonstrated a significantly reduced recurrence and duration of infection (outbreak) (57–58). The efficacy of topical application, together with in vitro results (48, 54), suggest that free zinc may indeed coat HSV virions, thus preventing infection. Further research into this molecular mechanism is warranted.

Apart from HCMV mentioned above, the effect of zinc on other members of the *Herpesviridae* family remains unknown because of a lack of clinical data. Mechanistically, zinc ions have been shown to inhibit Varicella-Zoster virus by inactivating free virus in vitro (59). Both HSV and Varicella-Zoster virus belong to the *Alphaherpesvirinae* subfamily, reflecting their genetic relatedness, and similar mechanism of inhibition.

TABLE 1 In vitro studies assessing the antiviral efficacy of zinc[1]

Virus	Antiviral effect	Zinc	Effective dose	Reference
Coronavirus	Inhibition of RdRp template binding and elongation	PT + $Zn(OAc)_2$	2–320 μM PT + 2–500 μM Zn	(60)
Encephalomyocarditis virus	Inhibition of viral polyprotein cleavage	$ZnCl_2$	0.4–1.5 mM	(61)
	Inhibition of viral polyprotein cleavage	$ZnCl_2$	0.1–1 mM	(62)
	Inhibition of viral polyprotein tertiary structure	PT, HK	5–20 μM PT, 60–125 μM HK	(63)
	Inhibition of viral polyprotein tertiary structure	PDTC	15–125 μM PDTC	(63)
Foot and mouth disease virus	Inhibition of viral polyprotein cleavage	$ZnCl_2$, $Zn(OAc)_2$	0.1–2 mM	(64)
	Inhibition of viral RNA and procapsid synthesis	$ZnCl_2$	10–150 μM	(65)
Hepatitis C virus	Inhibition of RNA polymerase	$ZnCl_2$	60–300 μM	(66)
	Inhibition of viral replication	$ZnCl_2$, $ZnSO_4$	50–150 μM	(67)
	Metallothionein-dependent inhibition of viral replication	$ZnSO_4$	50 μM	(34)
Herpes simplex virus	Viral protein synthesis	$ZnSO_4$	N/A	(53)
	Inhibition of viral DNA polymerase	$Zn(OAc)_2$	0.1–2 mM	(52)
	Free virus inactivation	$ZnSO_4$	0.1–6 mM	(48)
	Free virus inactivation	$Zn(Glu)_2$, $Zn(Lac)_2$	1–50 mM	(54)
	Inhibition of protein ubiquitination and NF-κB activity	PT	1.2–18.9 mM	(41)
Human immunodeficiency virus	HIV protease inhibition	Not listed	0.2–2 mM	(68)
	Inhibition of viral transcription and particle production	$ZnCl_2$	70–700 μM	(69)
	Inhibition of reverse transcriptase	$ZnCl_2$	25–800 μM	(70)
Human papilloma virus	Stimulates proviral transcription factor activity, reversed by EVER2	N/A	N/A	(19)
	Inhibition of viral protein E6 and E7 synthesis stimulating apoptosis	CIZAR	500–750 μM	(71)
Respiratory syncytial virus	Reduction in viral titer and plaque count	$ZnCl_2$, $Zn(OAc)_2$, $Zn(Lac)_2$	0.01–10 mM	(72)
Rhinovirus	Inhibition of viral polyprotein cleavage	$ZnCl_2$	100–800 μM	(73, 74)
	Inhibition of viral polyprotein cleavage	$ZnCl_2$	0.1–1.2 mM	(61)
	Inhibition of viral polymerase	not listed	>0.6 μM	(75)
	Inhibition of viral polyprotein processing	PT, HK	5–20 μM PT, 60–125 μM HK	(63)
	Inhibition of viral polyprotein processing	PDTC	15–125 μM PDTC	(63)
Semliki Forest virus	Inhibition of endosomal membrane fusion	$ZnCl_2$	25–100 μM	(76)
	Inhibition of endosomal membrane fusion	$ZnCl_2$	2 mM	(77)
Sindbis virus	Inhibition of viral particle production and polyprotein cleavage	$ZnCl_2$	0.1–1.8 mM	(78)
Transmissible gastroenteritis virus	Inhibition of viral RNA and protein synthesis	$ZnCl_2$, $ZnSO_4$	10–200 μM	(79)
Vaccinia virus	Inhibition of RNA synthesis and viral yield	$ZnSO_4$	100–300 μM	(80)
	Inhibition of viral particle production and polyprotein cleavage	$ZnCl_2$	50–400 μM	(81)
	Inhibition of viral topoisomerase	Not listed	2.5 mM	(82)
Varicella-zoster virus	Free virus inactivation	$Zn(Pic)_2$, $Zn(Asp)_2$	10 μM	(59)

[1] CIZAR, zinc citrate compound; HK, hinokitiol; N/A, not applicable; PDTC, pyrrolidine-dithiocarbamate; PT, pyrithione; RdRp, RNA-dependent RNA polymerate; $Zn(Asp)_2$, zinc aspartate; $ZnCl_2$, zinc chloride; $Zn(Glu)_2$, zinc gluconate; $Zn(Lac)_2$, zinc lactate; $Zn(OAc)_2$, zinc acetate; $Zn(Pic)_2$, zinc picolinate; $ZnSO_4$, zinc sulfate.

FOI 26-3132 - Document 5

TABLE 2 Human clinical studies using zinc as an antiviral therapy [1]

Viral infection/condition	Antiviral/therapeutic effect	Effective dose	Treatment	Reference
Torque teno virus	Reduced viral load following stem cell transplant	600 mg $ZnSO_4$/d	Oral	(83)
Herpes simplex	Reduced duration and severity of outbreak	ZnO/glycine cream (0.3% ionic Zn)	Topical	(57)
	Reduction in outbreak recurrence	0.025% $ZnSO_4$ solution	Topical	(84)
	Reduction in outbreak recurrence	1–4% $ZnSO_4$ solution	Topical	(58)
Experimental rhinovirus	Reduced duration of illness with $Zn(Glu)_2$ only	$Zn(Glu)_2$ (13.3 mg) or $Zn(OAc)_2$ (5/11.5 mg) lozenges, every 2–3 h/d	Lozenge	(85)
Common cold	Reduced symptom severity, frequency, and duration	ZGG lozenges containing 23 mg Zn, every 2 h/d	Lozenge	(86)
	Reduced symptom severity, frequency, and duration	ZGG lozenges containing 24 mg Zn, every 2–3 h/d (Max 8)	Lozenge	(87)
	Reduced duration of symptoms	ZGG lozenges containing 13 mg Zn, every 2 h/d	Lozenge	(88)
	Reduced symptom severity and duration	$Zn(OAc)_2$ lozenges each containing 9 mg Zn, every 2 h/d	Lozenge	(89)
	Reduced symptom severity and duration	$Zn(OAc)_2$ lozenges each containing 13 mg Zn, every 2–3 h/d	Lozenge	(90)
	No effect on duration or severity	$Zn(Glu)_2$ (13.3 mg) or $Zn(OAc)_2$ (5/11.5 mg) lozenges, every 2–3 h/d	Lozenge	(85)
	Reduced symptom severity and duration	$Zn(OAc)_2$ lozenges each containing 13 mg Zn, every 2–3 h/d	Lozenge	(91)
Viral warts	Improved clearance of warts after 1–2 mo	10 mg/ kg $ZnSO_4$ to a maximum dose of 600 mg/d	Oral	(92)
	Clearance of warts based on concentration of zinc used	3 × 5 or 10% $ZnSO_4$/d	Topical	(93)
	Improved clearance of warts after 1–2 mo	10 mg/ kg $ZnSO_4$ to a maximum dose of 600 mg/d	Oral	(94)
	No benefit	10 mg/ kg $ZnSO_4$/d	Oral	(95)
	Resolution of 88% of lesions after 6 wk/3 sessions	Up to 3 intralesional injections with 2% $ZnSO_4$	Injection	(96)
Laryngeal papillomatosis	Resolution of papillomatosis (2 case studies)	10 mg/ kg $ZnSO_4$/d	Oral	(97)
HIV	Reduced infection, increased CD4 T cell count	200 mg/d $ZnSO_4$/d	Oral	(98)
	Increased CD4 T cell count	45 mg $Zn(Glu)_2$ every 8 h for 15 d, then 15 mg for 15 d	Oral	(99)
	Reduced incidence of diarrhea	10 mg elemental zinc as $ZnSO_4$/d	Oral	(100)
	No benefit	25 mg/d $ZnSO_4$/d	Oral	(101)
Chronic hepatitis C virus	Enhanced response to IFN treatment	2 × 75 mg polaprezinc/d	Oral	(102)
	No benefit to IFN treatment response	5 × 78 mg $Zn(Glu)_2$/d	Oral	(103)
	Reduced serum AST, ALT, and ferritin	3 × 75 mg polaprezinc/d	Oral	(104)
	Reduced serum ALT and Th2 cells (%)	2 × 75 mg polaprezinc/d	Oral	(105)
	Reduced incidence of HCC (albumin-dependent)	2 × 150 mg polaprezinc/d	Oral	(106)

[1] ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; ZGG, zinc gluconate/glycine; $Zn(Glu)_2$, zinc gluconate; ZnO, zinc oxide; $Zn(OAc)_2$, zinc acetate; $ZnSO_4$, zinc sulfate.

FOI 26-3132 - Document 5

FOI 26-3132 - Document 5

Picornaviridae.

It was clear as early as 1974 that zinc possessed an inhibitory effect on picornovirus polyprotein processing (73). Before 1980, zinc inhibition of picornovirus proteases from human rhinovirus isolates (73, 74), encephalomyocarditis virus (62), poliovirus (61), and foot and mouth disease virus (64, 65) had all been demonstrated. More recent studies using zinc ionophores have illustrated that zinc interferes with the autocatalytic processing of the viral protease $3CD^{pro}$ into $3C^{pro}$ in the picornavirus coxsackievirus B3, thus inhibiting processing of the viral polyprotein (107). However, this was not the case for encephalomyocarditis virus, where zinc appeared to inhibit the tertiary structure within the viral polyprotein (107). Together, these data suggest that zinc may interfere with proteolytic processing of the viral polyprotein because of misfolding, or through direct actions on the viral protease $3CD^{pro}$.

Clinical studies using zinc supplementation are primarily limited to rhinovirus infection, and are often grouped with other "common cold" viruses such as influenza and coronaviruses. The majority of studies use zinc lozenges with various zinc formulations and concentrations, possibly explaining the large variability in results [extensively reviewed in (108) and (109)]. Importantly, the amount of ionic zinc present at the site of infection (oral and nasal mucosa) is highly correlated to the study outcome (51, 108), and is dependent on the zinc formulation. At a physiological pH and 37°C, zinc gluconate for example, releases high amounts of ionic zinc, whereas zinc aspartate releases none (108). Upon examining only the relevant studies where high doses of ionic zinc were used, a clear reduction in cold duration of 42% was calculated (109). Whether this was caused by viral inhibition, improved local immune response, or an amelioration of symptoms remains uncertain.

Other respiratory tract infections: influenza, coronavirus, and metapneumovirus.

Few studies have examined the antiviral effects of zinc on other respiratory viruses. In vitro replication of influenza (PR/8/34) is significantly inhibited by the addition of the zinc ionophore pyrrolidine dithiocarbamate (110), perhaps through inhibition of the RNA-dependent RNA polymerase (RdRp), as had been suggested 30 y earlier (111). In similar fashion, severe acute respiratory syndrome (SARS) coronavirus RdRp template binding and elongation was inhibited by zinc in Vero-E6 cells (60). Moreover, zinc salts were shown to inhibit respiratory syncytial virus, even while zinc was incubated with HEp-2 cells only before infection, and then removed (72). The authors suggest that this indicates an inhibitory mechanism similar to HSV by preventing viral membrane fusion; however, no measures were taken to assess changes in intracellular zinc content, nor inhibition of other aspects of the viral life cycle.

Flaviviridae: a focus on HCV.

Flaviviruses represent a number of insect-borne viruses including dengue and West Nile virus, as well as the hepatotrophic virus, HCV. The effect of zinc on insect-borne flaviviruses is scarce; however, in vitro studies by our group (34) and others (67) have demonstrated that zinc salts can reduce HCV replication (~50% at 100 μM $ZnSO_4$), perhaps by inhibiting the HCV RdRp, as shown in *E. coli* [half maximal inhibitory concentration (IC50) ~60 μM] (66). Although this is a potential mechanism, it has not been examined in eukaryotic cells in which zinc homeostasis is significantly different.

If left untreated, HCV becomes a chronic hepatic infection in around two-thirds of individuals (112), resulting in a significant reduction in plasma zinc (113). Consequently, zinc supplementation in HCV studies have focused on improved patient outcomes, particularly decreased liver inflammation, and enhanced response to antiviral treatment. Supplementation with 150 mg/d polaprezinc (a bioavailable zinc L-carnosine chelate) has been shown to reduce markers of hepatic inflammation alanine aminotransferase and aspartate aminotransferase alone (105), and in combination with the antiviral treatment IFN-α (106). Moreover, polaprezinc significantly improved the rate of viral clearance, particularly in patients with lower viral loads at baseline (102). The mechanisms underlying these observations remain uncertain; however, are likely a combination of direct antiviral effects and strengthening of the antiviral response. Zinc supplementation and the antiviral response is reviewed below.

Togaviridae.

Like flaviviruses, togaviruses primarily consist of arthropod-borne viruses such as Semliki Forest virus, Western equine encephalitis virus, and Chikungunya virus. Viral infection occurs by receptor-mediated endocytosis, followed by fusion of virus and endosomal membranes, and particle release into the cytoplasm (114). Using liposome (76), red blood cell (115), and BHK-21 (77) cell model systems, zinc has been shown to efficiently inhibit membrane fusion of Semliki Forest virus and sindbis viruses. Zinc ions interfere with membrane fusion by binding to a specific histidine residue revealed on the viral E1 protein at low endosomal pH (77). Unfortunately, the in vivo relevance of this model is unclear because of the high concentration of zinc (>1 mM) used. Notably, concentrated zinc is present in vesicular zincosomes that are thought to serve as intracellular zinc storage vesicles (116). Similar to the mechanism used by macrophages to inhibit intracellular *Mycobacterium spp*., zincosome fusion to viral endosomes may inhibit key aspects of the viral life cycle such as togavirus membrane fusion.

Retroviridae: HIV.

Retroviruses are named after their ability to transcribe RNA into DNA using their unique reverse transcriptase (RT), consequently allowing integration of retroviral DNA into the host genome. The integrated provirus can then establish a latent infection for the life of the host and is a major barrier to virus cure strategies, particularly for HIV-1 (117). Similar to viral RdRps, zinc has also been identified as

an inhibitor of retrovirus RTs (118, 119). Fenstermacher and DeStefano demonstrated in 2011 that Zn^{2+} cations can displace Mg^{2+} ions from HIV-1 RT, promoting the formation of an excessively stable, but incredibly slow and inefficient replication complex (70). Zinc was also shown to inhibit the HIV-1 protease in 1991 (68), and to inhibit viral transcription in 1999 (69), but has received little attention since, with the exception of molecular simulation experiments that identified the zinc-binding sites at the catalytic aspartate-25 residue (120). As stated above, HIV can also stimulate zinc influx into monocytes (33), which may appear contradictory based on its antiretroviral properties. Latently infected monocytes and macrophages, however, can act as viral reservoirs for HIV (121), and could therefore benefit from zinc-mediated inhibition of cell death. In fact, unlike the majority of $CD4^+$ T cells, low levels of replication in macrophages do not result in cell death (122), making them a viable reservoir, in addition to long-lived resting $CD4^+$ T cells, for viral recrudescence after cessation of antiretroviral treatment.

Zinc deficiency is common in HIV-infected individuals, where it is associated with inflammation (123), immunological failure (124), and death (125). A recent Cochrane Review examined the role of micronutrient supplementation in people living with HIV (126). Although a number of studies demonstrated beneficial effects of zinc supplementation, the majority were underpowered. The authors concluded that zinc supplementation probably increases blood zinc concentration (moderate certainty), and may increase $CD4^+$ counts (low certainty).

Unlike zinc supplements, prophylactic zinc gels have shown a substantial benefit to limit HIV infection in vivo. Complete protection against vaginal SHIV-RT (a simian HIV virus expressing the human RT) infection in macaques was obtained by pretreating animals with an antiviral gel containing 14 mM zinc acetate and 50 μM MIV-150, a reverse transcriptase inhibitor (127). When used alone, zinc acetate is a potent antiviral, providing 66% protection against SHIV-RT vaginal infection (56) and an EC50 <100 μM in peripheral blood mononuclear cells against a range of HIV strains (128). Importantly, zinc treatment did not affect viral titers in macaques that became infected, nor did it result in zinc resistant HIV mutants with conserved *pol* (RT) mutations. These data suggest that zinc may not interfere with the HIV RT, but instead inactivate free virus or prevent viral attachment/penetration as reported for HSV (48, 54).

Papillomaviridae.

HPVs are oncogenic viruses that infect basal epithelial cells, where they stimulate proliferation resulting in warts. Although cutaneous warts are usually self-limiting and harmless, mucosal strains of HPV (e.g. high risk HPV-16 and -18) are a primary cause of cervical cancers (129). HPV oncoproteins E6 and E7 in particular, are significant drivers of cell proliferation and resistance to cell death by stimulating the degradation of tumor suppressor p53 and pRb, respectively [reviewed in (130)]. Although nuclear zinc appears to enhance HPV replication (see ***Zinc homeostasis and viral infection***), exogenous zinc treatment (CIZAR, zinc chloride and citric acid anhydrous) can effectively inhibit production of viral oncogenic proteins E6 and E7 (71). The inhibition of E6 and E7 by zinc results in apoptosis of cervical carcinoma cells, as they regain the function of tumor suppressors p53 and pRb (71). The mechanism by which zinc downregulates E6 and E7 expression is unknown, but may be preceded by a zinc-driven blockade in another component of the viral life cycle.

It would appear that both topical and oral zinc supplementation strategies have proven tremendously effective for cutaneous and genital warts. Unfortunately, the vast majority of studies are either underpowered, lacking suitable controls, or single case studies. Nonetheless, a recent systematic review concluded that zinc supplementation was the most effective systemic treatment for cutaneous warts, when compared to other available options (131). It should be noted, however, that individuals with persistent viral warts are often zinc-deficient or have lower concentrations than their healthy counterparts (132). In fact, studies demonstrating the most significant responses to zinc treatment had engaged patients that were primarily zinc-deficient (>70 μg/dL) (92, 94). Nonetheless, 78% (94) and 100% (92) of patients showed clearance of lesions in response to oral zinc sulfate (10 mg/kg up to 600 mg/d) compared to 13% and 0% of the placebo group, respectively. Topical zinc formulations have also proved efficacious for treatment of viral warts. A small study using a 4-wk topical 10% zinc sulfate regimen for plane warts demonstrated an 86% response rate (6/7), compared to a 10% response rate (1/10) in the control group (93).

Recent work suggests that treatment of vaginal HPV infections with topical zinc formulations may benefit the millions of women that remain unvaccinated against HPV. A recent pilot study demonstrated that intravaginal infusion of 500 μM zinc citrate in women diagnosed with high-risk HPV resulted in a 64% clearance rate, compared to 15% in the control group (133). Additional studies in mice have demonstrated that MZC, a formulation containing MIV-50, zinc acetate, and carrageenan, efficiently inhibited vaginal and anorectal HPV-16 pseudoviral particle infection (134).

In summary, it is evident that zinc possesses antiviral properties against a number of viral species. Although mechanistic studies are lacking, zinc appears to inhibit viral protease and polymerase enzymatic processes, as well as physical processes such as virus attachment, infection, and uncoating (**Figure 1**). Unfortunately, these mechanisms have not been well scrutinized in clinical studies, where zinc may provide inexpensive and effective adjunct treatments for many viral infections.

The role of zinc in antiviral immune signaling

Ionic zinc possesses unique and distinct antiviral properties against a number of human viruses; however, the antiviral immune response led by IFNs is invariably required to clear infections. Zinc has been shown to contribute to a number of innate and adaptive immune signaling pathways that have

FOI 26-3132 - Document 5

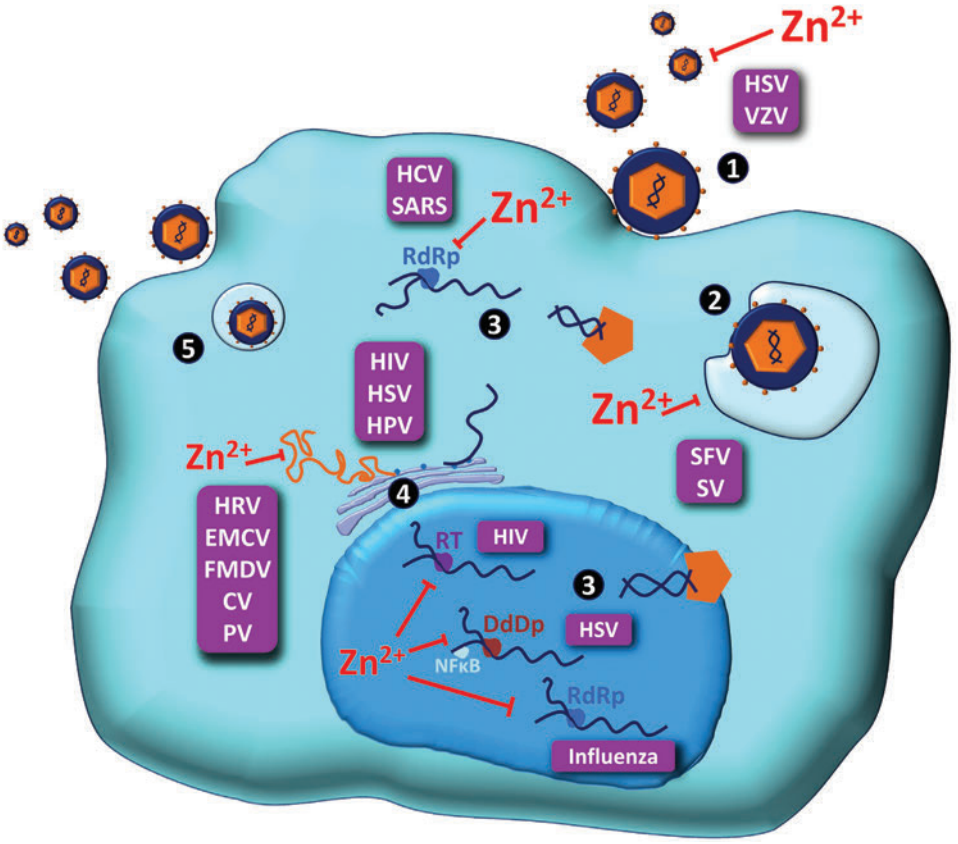


FIGURE 1 The diverse stages of viral replication cycles that are inhibited by zinc. In vitro studies have demonstrated a number of mechanisms by which zinc interferes with the viral replication cycle. These include free virus inactivation (1), inhibition of viral uncoating (2), viral genome transcription (3), and viral protein translation and polyprotein processing (4). No studies to date, however, have demonstrated zinc-mediated inhibition of virus assembly and/or particle release. CV, coronavirus; DdDp, DNA-dependent DNA polymerase; EMCV, encephalomyocarditis virus; FMDV, foot and mouth disease virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; HRV, human rhinovirus; HSV, herpes simplex virus; PV, polio virus; RdRp, RNA-dependent RNA polymerase; RT, reverse transcriptase; SARS, severe acute respiratory syndrome coronavirus; SFV, Semliki Forest virus; SV, sindbis virus; VZV, varicella-zoster virus; Zn, zinc.

been comprehensively reviewed recently (135). As such, this review will focus specifically on the role of zinc in the immune response to viruses.

Viral infections are recognized by a number of innate immune receptors termed pattern recognition receptors (PRRs). These include the cell surface and endosomal Toll-like receptors (TLRs), as well as a variety of cytosolic PRRs such as RIGI, MDA5, and IFI16 that primarily bind viral nucleic acids (136). Following ligand binding, PRRs share a number of downstream signaling intermediates, that ultimately activate both inflammatory (NF-κB, AP1) and innate immune (IRF1/3/7) transcription factors. These transcription factors cooperate to induce expression of IFNs, of which there are 3 types: type I (IFN-α and IFN-β), type II (IFN-γ), and type III (IFN-λs). Type I and III IFNs activate very similar antiviral signaling pathways; however, the type I IFN response is ubiquitous, whereas the type III IFN response is limited to a subset of immune cells, as well as epithelial cells of the liver, gastrointestinal, and pulmonary tracts (137). Although both IFN types bind unique receptors, they activate a common signaling cascade where STAT1 and STAT2 heterodimerize and bind IRF9, followed by translocation into the nucleus and subsequent binding of the IFN-sensitive response element that is present in hundreds of gene promoters. As stated previously, these ISGs possess numerous roles including immune cell chemotaxis and activation, as well as numerous antiviral mechanisms to inhibit viral replication within infected and neighboring cells.

Zinc and pathogen recognition.

Upon recognition of microbial antigens by TLRs, a rapid and transient influx of free zinc ions occurs. Interestingly, this has been demonstrated in response to viral stimuli, imiquimod, ssRNA40 (TLR7), and CpG (TLR9), but not polyI: C (TLR3) in the mouse macrophage RAW 264.7 cell line (138). In response to TLR7 activation, zinc was shown to reduce the production of type I IFNs and ISGs CD80

and CD86. Based on results using other stimuli, the authors suggest that zinc can inhibit IRF3-, and perhaps IRF7-dependent IFNB production, by limiting activation and/or nuclear translocation (138). The role of the zinc influx in this context remains undefined, but may reflect a regulatory mechanism to prevent excessive IFN production.

Although no direct inhibition of IRF signaling by zinc has been demonstrated, zinc can modulate a number of factors upstream of IRF activation. For example, the IκB kinase (IKK) members IKKα and IKKβ are inhibited by zinc, albeit at high concentrations of ∼0.5 μM (139). IKKα has been shown to activate IRF7 in response to TLR7/9 stimulation (140), whereas IKKβ (141), IKKε and TANK-binding kinase-1 (TBK1) (142) can activate IRF3 following TLR3 stimuli. Zinc can also stimulate expression of the deubiquitinating enzyme A20 (43) to inhibit the pathogen response. A20 is a regulator of NF-κB- (143), TLR3- (144), and RIGI-mediated (145) IFN production, most likely by targeting PRR signaling components TIR-domain-containing adapter-inducing interferon-β (TRIF), TNF Receptor Associated Factor (TRAF) 2, and TRAF6. A20-deficient cells are hyper-responsive to viral infection, possess increased activation of NF-κB, IRF3, and IRF7, and improved viral clearance (146).

Zinc and the interferon response.

After pathogen recognition, NF-κB, AP1, and IRF3/7 bind IFN promoters to stimulate type I/III IFN production. Zinc plays a significant role in the response to IFNs by modulating secretion, cytokine potency, and receptor binding, as well as influencing signaling intermediates and pathway inhibitors. A recent study has demonstrated that intracellular zinc can reduce IFN secretion by destabilizing *sortilin* mRNA transcripts (147). Sortilin is an endosomal protein that facilitates secretion of cytokines such as IFN-γ and IL6 (148), and its depletion results in a significant reduction in secretion of IFN-α. Consequently, because sortilin ensures trafficking and secretion of numerous cytokines, it is possible that zinc also inhibits the secretion of other IFNs.

Structural studies have demonstrated that zinc ions can mediate dimerization of IFNA molecules (149). Nonetheless, apart from crystallization studies, dimers were difficult to generate despite using concentrated IFN (50 μM) and zinc (1 mM). It is therefore likely that the circulating active form of IFN-α is monomeric. A single study performed in 2001 showed that zinc can increase the antiviral activity of IFN-α 10-fold against rhinovirus challenge (150). Although this study drew radical conclusions, antiviral activity was based on cytopathic effect alone, and its results have not been reproduced since. Moreover, zinc was added before viral infection, which is known to interfere with rhinovirus polyprotein processing (73, 74), as reviewed above.

Unlike type I IFNs, a recent study by our group has shown that zinc can inhibit IFN-λ3 signaling, most likely by preventing receptor binding and subsequent signaling (28). Upon demonstrating in 2014 that metallothionein expression was *IFNL* genotype-dependent, and inversely associated with ISG expression in HCV (151), we showed that serum zinc was the driver of hepatic metallothionein expression. Although zinc had minimal effect on IFN-α signaling, it could almost ablate IFN-λ3 signaling at a concentration of 50 μM, resulting in a significant reduction in its antiviral activity (28). Interestingly, we found no inhibition of IFN-λ1 activity using 50 μM $ZnSO_4$, suggesting a highly specific interaction. The mechanism by which zinc interferes with the IFN:receptor interaction remains uncertain; however, we have ruled out an effect of zinc on IFN-λ3 disulfide bond formation.

Type I and III IFNs bind to unique receptor complexes composed of IFN-α receptors IFNAR1/IFNAR2 and IFN-λ receptors IFNLR1/IL10RB, respectively, but signal via almost identical pathways. Consequently, zinc may act to reinforce the shared IFN signaling cascade by inhibiting protein tyrosine phosphatase enzymatic activity (152). Following receptor engagement by IFNs, intracellular Janus protein tyrosine kinases Jak1 and Tyk2 become phosphorylated, which in turn phosphorylate STAT molecules to stimulate ISG expression. By dephosphorylating these key signaling molecules, a number of phosphatases have been shown to "put the brakes" on IFN signaling. Phosphatases tyrosine-protein phosphatase non-receptor type 6 (SHP1), type 11 (SHP2), and protein phosphatase 2A (PP2A) have all been shown to inhibit JAK-STAT phosphorylation (153–155), and are all inhibited by zinc ions, predominantly in the nanomolar range (156–158). Interestingly, PP2A can also inhibit the phosphorylation of IRF3, thus regulating antigen recognition by PRRs (159). Conversely, the tumor suppressor phosphatase and tensin homologue (PTEN) stimulates IRF3 activation by removing inhibitory phosphorylation at Ser97 (160), and is also inhibited by zinc at nanomolar concentrations (161). Zinc inhibits numerous pro- and antiviral phosphatases, with the net effect on virus recognition and response being undefined, which clearly requires further study.

To enable a highly controlled IFN response, negative regulators of IFN signaling are often ISGs. These include the suppressors of cytokine signaling (SOCS-1 and SOCS-3), which bind and inhibit JAK protein signaling, thus preventing signaling from numerous inflammatory (IL-6) and antiviral stimuli (162). Interestingly, zinc-driven activation of the MTF-1 transcription factor can induce expression of SOCS-3 in HepG2 cells (163). The zinc importer ZIP-14, which is responsible for zinc influx following inflammatory stimuli, was required for SOCS-3 expression, and may represent yet another zinc-mediated mechanism to limit the inflammatory response. Although the transporter responsible for hepatic zinc influx following IFN stimulation remains unknown, it is perceivable that ZIP-14 may drive zinc influx and subsequent SOCS-3 expression.

Zinc deficiency caused by disease, age, and lifestyle factors: lessons from supplementation

Zinc status is primarily determined by dietary zinc intake; however, additional factors such as dietary composition, alcohol intake, and disease state can significantly reduce zinc uptake and storage, or increase zinc excretion (164). With respect to dietary composition, zinc supplementation as part of a meal can significantly reduce zinc absorption when compared to water-based solutions of zinc (164). Moreover, dietary phytate, a natural chelator of zinc ions that is present in corn, rice, and cereals, can severely restrict zinc absorption (165). Consequently, diets containing high phytate: zinc molar ratios, can result in zinc deficiency, even with adequate zinc intake. Unfortunately, rural diets in low-income nations are often zinc-poor and phytate-rich because of a dietary reliance on rice and vegetables.

Aged individuals are also significantly more susceptible to zinc deficiency, increasing their likelihood of acquiring life-threatening viral infections (166). Ex vivo, zinc supplementation has been shown to improve leukocyte IFN-α production (167) and to reduce mononuclear cell TNF production (168). Year-long supplementation with 45 mg elemental zinc/d in elderly subjects (aged 55–87 y), has also demonstrated a dramatic reduction in the incidence of infection as well as plasma oxidative stress markers (168).

Alcoholism can stimulate severe zinc deficiency developed via numerous sociological and physiological mechanisms, with factors including but not limited to *1*) increased urinary zinc excretion (169), *2*) reduced zinc intake (poor diet) (170), *3*) reduced zinc absorption (171), and *4*) and a reduction in hepatic zinc stores (172). Alcohol also stimulates microbial dysbiosis and gastrointestinal permeability (173), a phenotype that can increase the likelihood of viral infection in the gut (174). Importantly, dietary zinc supplementation can improve intestinal barrier dysfunction as a result of alcohol and microbial infection (175, 176).

As previously discussed, zinc deficiency is common among chronic infections such as HPV, HCV, and HIV (113, 123). Consequently, a number of studies have examined the effects of zinc supplementation on antiviral immunity, inflammation, and treatment response. As described above, zinc supplementation can improve HCV treatment response and liver inflammation caused by chronic infection. In addition, long-term zinc treatment over 7 y has been shown to reduce the risk of hepatocellular carcinoma progression in chronic HCV patients, as assessed by multivariate analysis, compared to controls ($P < 0.05$) (105). Zinc supplementation has also been assessed as an adjunct therapy to antiretroviral administration in patients with HIV. One study reported a 4-fold reduction in the rate of immune failure, as well as decreased diarrhea in patients treated with zinc compared to controls ($P < 0.05$ for both groups) (124). A more recent study revealed an increase in $CD4^+$ T cell count in patients treated with a combination of zinc and antiretroviral therapy, compared to patients on antiretroviral therapy alone ($P < 0.05$) (177). Taken together, these data indicate that zinc deficiency is associated with greater disease activity in the context of chronic viral infection. Oral zinc supplementation may act in a synergistic manner when co-administered with antiviral therapy and contribute to improved clinical outcomes.

Vaccination studies.

Zinc supplementation during vaccination strategies has provided an opportunity to examine the role of zinc in the humoral response to viruses. A particular focus has been applied to the effect of zinc supplementation on rotavirus vaccination because of the high rate of mortality associated with childhood diarrhea in developing countries. Unfortunately, although zinc deficiency is associated with increased risk of rotavirus gastroenteritis (178), it does not greatly increase the development of humoral immunity followed by vaccination (rotarix), as defined by seroconversion rate (179). Nonetheless, a pooled analysis of randomized trials performed in 2000, demonstrated that zinc supplementation shortens the length of diarrheal episodes and reduced the rate of treatment failure or death by 42% in zinc-deficient children (180).

Comparable studies of supplementation with zinc before vaccination have produced similar disappointing results. Zinc supplementation did not improve seroconversion following administration of the oral poliovirus vaccine in infants (181), nor did it improve the immunological response to HBV (182) or influenza vaccination (183) in the elderly. Although there remains little evidence that zinc improves viral vaccination responses, a small number of studies suggest that zinc may improve antibody titers and antibacterial responses to pneumococcus (184) and cholera infections (185).

Conclusions and Future Perspectives

The tight regulation of zinc homeostasis both systemically and intracellularly indicates that zinc plays an essential role in human health. Although zinc is a component of ~10% of the human proteome, zinc in different forms (free compared with protein-bound) can stimulate a variety of signaling events, including the antiviral response. In vitro studies suggest that free zinc may possess potent antiviral effects, and are supported by trials of creams, lozenges, and supplements with high free zinc content. Moreover, zinc-binding proteins such as the metallothioneins may possess antiviral roles, although their specific function remains uncertain. Nonetheless, zinc treatment applied at a therapeutic dose and in the right form has the potential to drastically improve the clearance of both chronic and acute viral infections, as well as their accompanying pathologies and symptoms. Consequently, the role of zinc as an antiviral can be separated into 2 categories: *1*) zinc supplementation implemented to improve the antiviral response and systemic immunity in patients with zinc deficiency, and *2*) zinc treatment performed to specifically inhibit viral replication or infection-related symptoms (75, 78–82, 83, 85–91, 95–101, 103, 104).

Acknowledgments

The authors' responsibilities were as follows—SR and GA designed the review, all authors performed the study selection, all authors read and approved the final manuscript.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

Naunyn-Schmiedeberg's Archives of Pharmacology (2020) 393:1153–1156
https://doi.org/10.1007/s00210-020-01902-5

BRIEF COMMUNICATION

Ivermectin, antiviral properties and COVID-19: a possible new mechanism of action

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Received: 29 April 2020 / Accepted: 10 May 2020 / Published online: 27 May 2020
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Abstract

Ivermectin is an antiparasitic drug that has shown also an effective pharmacological activity towards various infective agents, including viruses. This paper proposes an alternative mechanism of action for this drug that makes it capable of having an antiviral action, also against the novel coronavirus, in addition to the processes already reported in literature.

Keywords Ivermectin · Antiviral · Ionophore · COVID-19 · SARS-CoV-2

Ivermectin [mixture of 22, 23-dihydroavermectin B1a (80%) and 22, 23-dihydroavermectin B1b (20%)] (Fig. 1) is a macrocyclic lactone with a broad-spectrum antiparasitic pharmacological activity (Gonzalez Canga et al. 2008). It is the safest and most effective semi-synthetic derivative of the entire class of avermectins, discovered in 1975 by Professor Satoshi Ōmura as fermentation products of the actinomycete bacterium *Streptomyces avermitilis* (Crump 2017) (later reclassified in *S. avermectinius* (Takahashi et al. 2002)). Its main pharmacodynamics is to bind some channel proteins for chlorine controlled by glutamate, typical of specific classes of invertebrates, causing a greater permeability to this electrolyte: all this causes a hyperpolarization of the cell membrane, blocking inhibitory neurotransmission in neurons and myocytes, resulting in paralysis and death (Geary 2005). Commercialized since 1981, its low cost, its high efficacy and safety, and the marked tropism for helminths (therefore with an almost zero impact on the biochemistry of human beings) have led to its inclusion in the twenty-first World Health Organization's List of Essential Medicines (World Health Organization 2019).

Ivermectin is a versatile drug with unique characteristics, which make it interesting also for basic and applied research (in particular for drug repurposing): it seems to reveal an antibacterial (Lim et al. 2013; Ashraf et al. 2018), antiviral, and anticancer activity (Juarez et al. 2018; Intuyod et al. 2019), besides being potentially useful for the treatment of some chronic pathologies (Ashraf and Prichard 2016; Ventre et al. 2017), result of an action on a wide range of cellular targets.

Regarding its role as an antiviral agent, its efficacy has been demonstrated on several viruses, both in vitro and in vivo. Among the many mechanisms by which it performs its function, the most consolidated one sees ivermectin as an inhibitor of nuclear transport mediated by the importin α/β1 heterodimer, responsible for the translocation of various viral species proteins (HIV-1, SV40), indispensable for their replication (Wagstaff et al. 2011; Wagstaff et al. 2012). This inhibition appears to affect a considerable number of RNA viruses (Jans et al. 2019; Caly et al. 2012), such as Dengue Virus 1-4 (DENV) (Tay et al. 2013), West Nile Virus (WNV) (Yang et al. 2020), Venezuelan Equine Encephalitis Virus (VEEV) (Lundberg et al. 2013), and Influenza (Gotz et al. 2016). In addition, ivermectin has been shown to be effective against the Pseudorabies virus (PRV, with a DNA-based genome), both in vitro and in vivo (Lv et al. 2018), using the same mechanism. Caly et al. (Caly et al. 2020) have recently shown that the drug also inhibits the replication of the SARS-CoV-2 virus in vitro, however not clarifying how it occurs. Since the causative agent of COVID-19 is an RNA virus, it can be reasonably expected an interference with the same proteins and the same molecular processes described above.

However, ivermectin could prove to be a powerful antiviral, therefore also useful for a possible treatment of the new coronavirus associated syndrome, even from a new perspective. This could happen assuming its role as an ionophore agent, only hinted in the recent past but never fully described (Juarez et al.

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FOI 26-3132 - Document 5

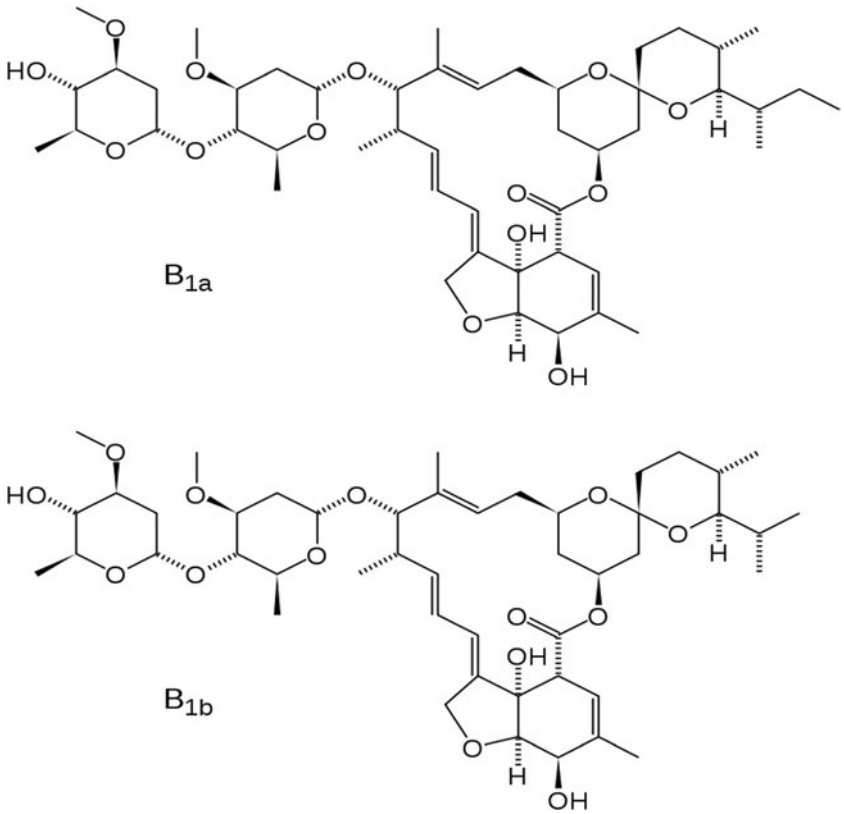


Fig. 1 Structural formulas of ivermectin compounds

2018). Ionophores are molecules that typically have a hydrophilic pocket which constitutes a specific binding site for one or more ions (usually cations), while its external surface is hydrophobic, allowing the complex thus formed to cross the cell membranes, affecting the hydro-electrolyte balance (Freedman 2012). These chemical species have historically been used to study the mitochondrial respiratory chain and ATP synthesis in eukaryotes (in this case also known as decoupling agents, such as 2, 4-dinitrophenol), and their antibiotic activity has long been appreciated (Bakker 1979). It is also hypothesized their role as antiviral drugs (Krenn et al. 2009; Sandler et al. 2020) and anticancer chemotherapeutic agents (Kaushik et al. 2018). Thinking of the structure of two of the most important ionophores, monensin A and valinomycin, respectively a polyether and a depsipeptide antibiotic, it is clear that they internally present many oxygen atoms (with related free electron doublets), indispensable for binding cations and transporting them through phospholipidic bilayers.

At a first glance, the two structures that make up the ivermectin formula do not have these chemical properties, nor those mentioned above, essential for a compound to be defined as ionophore. However, it can be hypothesized that two ivermectin molecules, reacting with each other in a "head-tail" mode, can create a complex suitable to be considered such (Fig. 2). This interaction could occur spontaneously or be mediated by the binding of the same molecules to some plasma transport proteins, in particular albumin (Klotz et al. 1990), which would have the role of positioning them in the correct way to obtain the proposed configuration.

As it can be seen, in this way, an internal cavity is formed: the oxygen atoms (indicated in red), now present in greater number, work as Lewis bases and could therefore coordinate a series of cations (Lewis acids). On the other hand, the –OH groups are highlighted in blue and they could have a decisive role in the stabilization of the new structure, with the establishment of chemical bonds between these functional groups: one or more –O– bridges (however, it is difficult the formation of ether bonds, since acid catalysis at high temperature is not possible under normal conditions, both in vitro and in vivo) or more probably hydrogen bonds could be formed, even among more molecular complexes of this type. However, the formation of other weak and strong interactions of various kinds cannot be excluded. Otherwise, specific cations could bind the two molecules in the proposed way, creating themselves the final structure and stabilizing it: there are examples already known in literature (Abbott et al. 1979). The external part of the complex, then, would already have in itself all the hydrophobic characteristics necessary to carry ions through the viral membrane. As a consequence, it would be determined an ionic imbalance between the external and internal environment, with the recall of water and consequent osmotic lysis. This would allow to neutralize the virus at an early stage of the

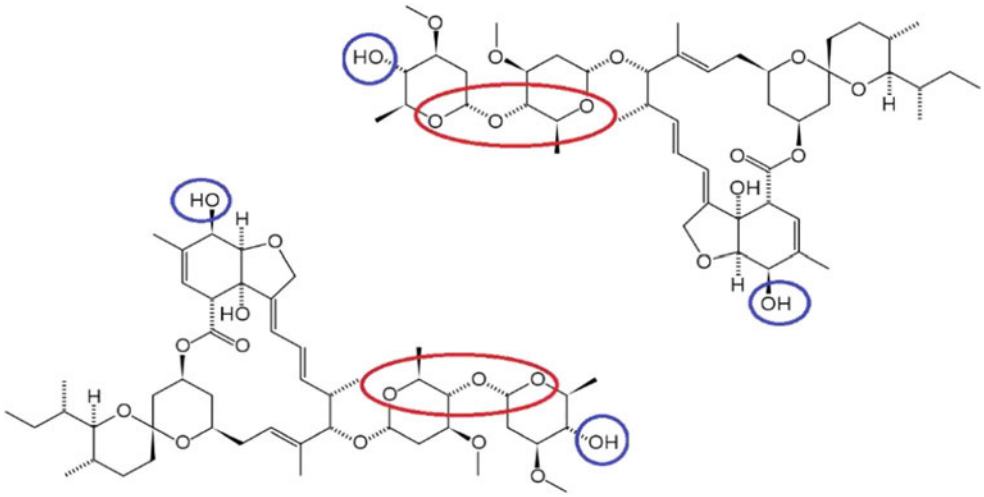


Fig. 2 Possible interaction mechanism between two ivermectin molecules

infection, before; therefore, it can adhere to the host cells and enter it to exploit their biochemical machinery for the production of other viral particles. However, this hypothesis would concern only viruses without a proteic capsid, a structure that shows a certain resistance to osmotic pressure, even if to a lesser extent than a bacterial, fungal, or plant cell wall (Cordova et al. 2003). The new coronavirus is one of these, presenting only a phospholipid envelope in defense of the genetic material, where its few proteins are inserted and which it acquires in the act of exiting the infected cells (Sigrist et al. 2020). This unconventional electrolyte uptake mode could also affect the potential of the viral membrane, threatening its integrity and functionality. The same goes for the viral proteins present here. Furthermore, the concentration variation of some cations, thus determined, could inhibit some key enzymes in the viral replication, such as RNA-dependent RNA polymerases (RdRp) (te Velthuis et al. 2010), already used as pharmacological targets.

Another indication in favor of a possible ionophore role for ivermectin comes from the analysis of molecular similarity that can be carried out through the Drugbank database (www.drugbank.ca). By setting a minimum similarity threshold for ivermectin equal to 0.7, about 14 results are obtained. Among the various selected molecules, the majority of which have antiparasitic and antibiotic activity (already not only on the market but also in the study and experimentation phase), a compound that has high structural similarity is nystatin (score of 0.72), an antimycotic drug with an ionophoric activity at the plasma membrane level, where it forms channels (Yamasaki et al. 2011; Stillwell 2016; Rang 2015).

Immediately afterwards, with a slightly lower similarity, it can be find amphotericin B and natamycin, all pharmacological molecules of assured ionophoric activity (score of 0.71 and 0.706, respectively) (Stillwell 2016; Rang 2015; Ramos 1989; Ikehara et al. 1986).

In conclusion, pending computational simulations and chemical-physical laboratory analysis, this hypothesis could be applied to other known pharmacological molecules, in order to identify compounds with probable ionophore nature to be used in research and clinical practice.

Authors' contributions All research phases (idea, drafting of the paper, and proofreading) were conducted by the only author, ER.

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FOI 26-3132 - Document 5

Zinc supplementation reduced morbidity, but neither zinc nor iron supplementation affected growth or body composition of Mexican preschoolers[1–3]

Jorge L Rosado, Patricia López, Elsa Muñoz, Homero Martinez, and Lindsay H Allen

See corresponding editorial on page 160.

ABSTRACT In rural Mexico and in many developing countries micronutrient deficiencies, growth stunting, and morbidity from infectious diseases are highly prevalent in young children. We assessed the extent to which growth stunting could be reversed and the number of infectious disease episodes reduced by zinc and/or iron supplementation. In a double-blind, randomized community trial 219 Mexican preschoolers were supplemented with either 20 mg Zn as zinc methionine, 20 mg Fe as ferrous sulfate, 20 mg Zn + 20 mg Fe, or a placebo. After 12 mo, plasma zinc increased significantly in the two zinc-treated groups, and plasma ferritin was significantly higher in the two iron-treated groups. There was no effect of treatments on growth velocity or body composition. Children in both zinc-supplemented groups had fewer episodes of disease (zinc alone, 3.9 ± 0.3; zinc + iron, 3.7 ± 0.4; placebo, 4.6 ± 0.5; $P < 0.03$), including diarrhea (zinc alone, 0.7 ± 0.1; zinc + iron, 0.8 ± 0.1; placebo, 1.1 ± 0.2; $P < 0.01$). Zinc and zinc + iron supplements reduced morbidity but had no effect on growth or body composition. *Am J Clin Nutr* 1997;65:13–9.

KEY WORDS Zinc, iron, Mexico, supplementation, growth, body composition, morbidity, children

INTRODUCTION

Undernutrition is manifested in most developing countries by early growth stunting and a high prevalence of micronutrient deficiencies. National surveys in Mexico showed that 25–50% of preschool children in rural areas are stunted (1), and that anemia affects 16–51% of urban children and ≤ 91% of those in poorer rural regions (2, 3).

The present study was conducted in the Valley of Solís, a rural region in central Mexico, as a logical intervention based on previous information about the nutritional status of children and adults in the valley. The children have been of normal weight and length when born, but compared with international reference values length *Z* scores fall immediately after birth, and weight *Z* scores start to fall at ≈3 mo of age (4). This growth faltering continues until 22 mo of age after which time growth rate is comparable with international reference values. Similar growth patterns have been reported in studies in other countries (5–7). The early growth stunting is likely to persist through adolescence if children remain in the same location (8) but may be at least somewhat reversed if diet and environmental conditions are improved (9, 10).

The causes of early growth stunting are not yet understood (11), but may include a nutritionally inadequate diet as well as clinical (12) or subclinical (13) infections. Although growth stunting has been traditionally attributed to protein-energy malnutrition, it is widespread even where protein and energy intakes are adequate (5, 6, 11) such as in Solís (14). Stunting is associated with habitual consumption of a diet that is low in animal products and accompanying micronutrients, and high in plant constituents such as phytate that inhibit the absorption of minerals (15, 16). We showed previously that the absorption of both zinc and iron is lower from a rural Mexican diet consisting primarily of maize than from a more refined urban Mexican diet (17). Because zinc supplementation has improved the growth and/or body composition of stunted children in countries such as the United States, Canada, Ecuador, China, and Guatemala (11, 18), the present study was designed to measure the effect of zinc supplementation on the growth and body composition of Mexican preschoolers. Unlike most of the previous zinc-supplementation studies reported in the literature, iron supplements were also provided because of the high prevalence of iron deficiency in this community (19). There is some, albeit limited, evidence that iron deficiency causes poor appetite and growth stunting (20), so we were concerned that simultaneous iron deficiency might have limited any zinc-induced growth response.

Both zinc and iron deficiency cause impaired immune response (21, 22), but it remains to be determined whether community-level supplementation with zinc or iron will reduce morbidity in marginally malnourished populations. We

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[2] Supported in part by US Department of Agriculture grant 90–37200-5478 (to LHA at the University of Connecticut), and grants 0810 and M9110 from CONACYT and NUTR97 from InterHealth Co, Concord, CA (to JLR at the Instituto Nacional de la Nutricion, Mexico City).

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Received February 13, 1996.

Accepted for publication August 8, 1996.

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therefore measured morbidity as an outcome of zinc and iron supplementation and as a potential confounder of the effect of supplementation on growth and body composition.

SUBJECTS AND METHODS

Subjects and location

The study was conducted in five rural communities in the Valley of Solís, located in the central highland plateau of the state of Mexico ≈150 km northwest of Mexico City. The communities ranged in size from 700 to 1500 individuals, or from ≈100 to 214 households. A census revealed that 290 children met our eligibility criterion of being between 18 and 36 mo old. The mothers of all of these children were invited to attend a meeting during which they were informed about the study purpose and protocol and potential risks and benefits. The mothers of 219 children attended the meeting, agreed to participate, and signed consent forms. This number of children met our goal of 48 per treatment group (192 total), which would have detected a difference of 1 cm in height with 80% power, assuming the SD of height change would be 1.5.

The protocol was approved by the Committee on Biomedical Research in Human Subjects of the National Institute of Nutrition, Mexico, and the Committee on the Use of Human Subjects in Research at the University of Connecticut.

Zinc and iron supplementation

Children were stratified by age and sex, and ranked by height, then they were randomly assigned to one of four treatment groups within each stratum. Ages were confirmed by birth certificates.

The four groups received a daily supplement consisting of 20 mL of a solution containing either 20 mg elemental Zn as zinc methionine, 20 mg Fe as ferrous sulfate, 20 mg Zn + 20 mg Fe, or a placebo (solution alone). Zinc methionine (Inter Health Co, Concord, CA) was chosen as the zinc source after we showed that it was better absorbed than other sources of zinc (23) and theoretically would reduce the risk of the zinc being bound by dietary phytate in the intestine. Ferrous sulfate was selected because it is the form of iron that is most commonly used for supplementation. Both the zinc and the iron salts were dissolved in a solution to disguise their bad taste and to ensure similar appearance. This solution contained sugar, citric acid, water, and artificial flavor (either orange or lemon). Before the study, the acceptability of the supplements was tested by sensory evaluation in children of the same age. The solutions were coded in such a way that their content was unknown to any of the project personnel, and the code was not broken until the end of data analysis.

Children in each group received the supplements for 12 mo. They were visited in their homes from Monday through Friday by a fieldworker who gave the supplement and ensured that it was completely consumed in her presence. The flavor of the supplement (lemon or orange) was changed every week to improve compliance. Compliance was excellent; the supplements were consumed on average on 97% of the days they were administered. Only 25 children were dropped from the study before the end of the 12 mo, primarily because of a changing family situation. Data from these children were not used in the statistical analyses.

Anthropometry

Anthropometric data were collected at baseline (before supplementation) and within 2 wk after 6 and 12 mo of supplementation. Measurements on all three occasions were performed by the same examiner (PL) following standard procedures (24). Measures included weight to 0.1 kg on a pediatric scale, standing height to 0.1 cm with an anthropometer, midupper arm circumference (MAC) to 0.1 cm by using a fiberglass tape measure on the left arm, and triceps skinfold thickness accurate to 0.1 mm by using a Lange skinfold caliper.

Clinical examinations and morbidity data

A clinical exam was conducted by a physician in the field clinic at baseline and at 6 and 12 mo after supplementation. Data collected during this exam included signs of nutrient deficiencies or other diseases. Any current diseases received appropriate treatment. No clinical symptoms of micronutrient deficiencies were apparent, and no child was dropped from the study on the basis of the relatively minor diseases that were detected.

Morbidity of the preschoolers was evaluated twice weekly by a questionnaire administered by a trained fieldworker who visited each child at home. Information was obtained from the child's mother about the presence or absence of illness on the day of the visit and since the previous visit. Any illness was recorded by the fieldworker on a precoded list that included the most common diseases and their symptoms, as well as the date when the symptoms started and the date when they disappeared. When the fieldworker had any doubt about the diagnosis or course of the disease, the physician in charge of the field clinic visited the child to confirm the diagnosis. Only data on infectious diseases were analyzed, with symptoms classified by a physician into the following categories: upper and lower respiratory disease (combined because there were only four episodes of lower respiratory disease), diarrhea, fever, and "other." "Respiratory disease" was defined as having any symptom such as runny nose, common cold, sore throat, or cough. "Diarrhea" was defined by the mothers based on their observation of frequent, loose stools. In this study and in our previous research in these communities we found a strong association between mothers' reporting and the physician's diagnosis of diarrhea (19). "Fever" was based on maternal reporting.

Biochemical indicators of zinc and iron status, and other nutrient deficiencies

A 2-mL sample of venous blood was collected from every preschooler at baseline and at 6 and 12 mo after supplementation. After the children had fasted overnight, blood samples were collected in an evacuated container and transferred to a tube that contained 0.05 mL sodium citrate as an anticoagulant. Duplicate hemoglobin and hematocrit determinations were performed within 3 h of blood collection. Hemoglobin was determined by using a Coulter counter (Coulter Electronics, Hialeah, FL). Plasma and red blood cells were dispensed as aliquots and frozen at −70 °C until analyzed. Plasma zinc was determined in duplicate by atomic absorption spectrophotometry, and plasma ferritin was measured by using a solid-phase radioimmunassay (Coat-A-Count Ferritin IRMA; Diagnostic

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Products, Los Angeles) and an international standard obtained from the World Health Organization Internal Laboratory for Biological Standards (National Institute of Biological Standards and Control, Hertsfordshire, United Kingdom). To detect infections or inflammation, plasma C-reactive protein (CRP) was measured by a Behring laser nephelometer after adding antiserum for CRP (Behring Diagnostics, Inc, Somerville, NJ); a protein control serum was included in the test (Behring Diagnostics, Inc).

Statistical analysis

All analyses were conducted by using SAS software for microcomputers. Analyses of the differences among the groups, and between each treatment group and the placebo group, were tested by analysis of covariance using initial values as covariates. *Z* scores were calculated from National Center for Health Statistics (NCHS) reference values for weight and height (25). Midarm fat area (MAFA) and midarm muscle area (MAMA) were derived from MAC and triceps-skinfold-thickness measurements (26). Changes in anthropometric measures were also analyzed separately for children who were stunted [height-for-age *Z* score (HAZ) < -2.0]. Morbidity data, combined from the entire 12 mo of the study because of the relatively low prevalence of illness, were analyzed by a two-way nonparametric analysis of variance and significant differences among groups were compared by the Kruskal-Wallis test (27). A P value < 0.05 was considered significant.

RESULTS

Assessment of growth and body composition

The characteristics of subjects in each group are shown in **Table 1**. No characteristic differed significantly among treatment groups. The children were growth stunted, with a mean (± SEM) HAZ of -1.6 ± 0.8. However, as expected, their weight-for-height was normal on average, showing that wasting was not a general problem in the group. No sex differences in anthropometry were found initially or in response to treatment, so that data are presented for the sexes combined.

Changes in weight, height, and *Z* scores after the 12 mo of intervention are shown in **Table 2**. The average linear growth rate varied from 8.9 to 9.3 cm/y among the treatment groups and was unaffected by type of treatment. In addition, in the three nutrient-supplemented groups final *Z* scores for weight-for-age (WAZ) and weight-for-height (WHZ) were similar to those in the placebo group. The growth velocities among treatments for only those children with an initial HAZ < -2.0 are also compared in Table 2. Although the group supplemented with zinc alone had a faster rate of height growth and a larger improvement in HAZ than the other groups, these differences were not significant. Similar results were found when all anthropometric data were analyzed 6 mo after the start of supplementation (not reported). We also analyzed the growth response of children with plasma zinc concentrations < 10.7 μmol/L at baseline, and found it to be unaffected by any treatment.

Changes in indicators of body composition after 12 mo of intervention are shown in **Table 3**. There were no significant differences among groups in any of the body composition indicators. This lack of treatment effect on body composition persisted when data were analyzed separately for children with an initial HAZ < -2.0 (Table 3), and when data were analyzed after the first 6 mo of intervention or for the group with initial plasma zinc < 10.7 μmol/L (data not shown).

Biochemical responses to supplementation

Changes in hemoglobin and biochemical indicators of nutritional status after 12 mo of intervention are described in **Table 4**. Both iron-supplemented groups (ie, iron alone, and zinc + iron) responded to supplementation with a highly significant increment in ferritin, whereas there was no increase in ferritin in the placebo- or zinc-supplemented groups. The prevalence of iron deficiency (plasma ferritin < 12 μg/L) was 43–57% in the four groups at baseline, falling 12 mo later to 24% and 23% in the placebo and zinc groups, respectively, and to zero in the two iron-supplemented groups. The mean hemoglobin concentration increased in all groups during the 12 mo, regardless of iron supplementation.

Plasma zinc increased significantly in the zinc- and zinc + iron–supplemented groups over the 12 mo, indicating that the supplemental zinc was absorbed. The mean prevalence of low plasma zinc (< 10.7 μmol/L) was 20% at baseline for all children combined. After 12 mo of supplementation the prevalence of low zinc concentrations was 27% and 21% in the placebo and iron-supplemented groups, respectively, but fell to 14% in the group supplemented with zinc alone and to 8% after supplementation with both zinc and iron. The children who had

TABLE 1
Characteristics of subjects in the four groups at the beginning of the study[1]

	Placebo (n = 55)	Iron (n = 53)	Zinc (n = 54)	Zinc + iron (n = 55)
Age (mo)	28.9 ± 1.06	27.5 ± 0.94	28.4 ± 1.02	28.8 ± 1.2
Height (cm)	83.1 ± 0.74	82.8 ± 0.89	83.2 ± 0.80	83.9 ± 0.99
Weight (kg)	11.1 ± 0.20	10.8 ± 0.23	11.1 ± 0.20	11.4 ± 0.27
MAC (cm)	14.6 ± 0.12	14.4 ± 0.15	14.4 ± 0.22	14.8 ± 0.16
TST (mm)	11.2 ± 0.31	10.4 ± 0.30	10.3 ± 0.27	11.3 ± 0.34
HAZ	−1.8 ± 0.12	−1.6 ± 0.16	−1.6 ± 0.14	−1.5 ± 0.13
WAZ	−1.4 ± 0.08	−1.6 ± 0.12	−1.4 ± 0.12	−1.2 ± 0.12
WHZ	−0.4 ± 0.08	−0.7 ± 0.08	−0.4 ± 0.08	−0.3 ± 0.12

[1] $\bar{x}$ ± SEM. MAC, midupper arm circumference; TST, triceps skinfold thickness; HAZ, height-for-age *Z* score; WAZ, weight-for-age *Z* score; WHZ, weight-for-height *Z* score. *Z* scores were calculated from National Center for Health Statistics reference values (25).

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TABLE 2
Effects of treatments on growth velocity and change in *Z* scores over 12 mo in subjects in the four groups[1]

	Placebo	Iron	Zinc	Zinc + iron
All children[2]				
Height (cm/y)	9.1 ± 0.22	9.0 ± 0.19	9.3 ± 0.21	8.9 ± 0.19
Weight (kg/y)	2.4 ± 0.07	2.4 ± 0.07	2.4 ± 0.07	2.3 ± 0.07
HAZ	0.13 ± 0.06	0.02 ± 0.06	0.16 ± 0.05	0.07 ± 0.06
WAZ	0.25 ± 0.05	0.28 ± 0.06	0.26 ± 0.05	0.16 ± 0.06
WHZ	0.29 ± 0.06	0.36 ± 0.06	0.25 ± 0.06	0.19 ± 0.08
Children with initial HAZ < −2.0[3]				
Height (cm/y)	9.2 ± 0.34	9.0 ± 0.28	9.6 ± 0.37	9.1 ± 0.32
Weight (kg/y)	2.3 ± 0.11	2.4 ± 0.11	2.4 ± 0.11	2.1 ± 0.16
HAZ	0.32 ± 0.09	0.19 ± 0.08	0.39 ± 0.09	0.31 ± 0.08
WAZ	0.26 ± 0.08	0.46 ± 0.10	0.38 ± 0.08	0.17 ± 0.14
WHZ	0.16 ± 0.08	0.45 ± 0.09	0.23 ± 0.11	0.03 ± 0.19

[1] $\bar{x}$ ± SEM. There were no significant differences between groups. MAC, midarm circumference; HAZ, height-for-age *Z* score; WAZ, weight-for-age *Z* score; WHZ, weight-for-height *Z* score.
[2] n = 47 in the placebo, 50 in the iron, 48 in the zinc, and 49 in the zinc + iron group.
[3] n = 19 in the placebo, 17 in the iron, 18 in the zinc, and 17 in the zinc + iron group.

low plasma zinc after supplementation had values < 9.2 μmol/L.

Morbidity response

The effect of supplementation on the number of diarrhea and respiratory disease episodes is shown in **Table 5**. The total number of disease episodes was substantially lower in both groups supplemented with zinc, although the reduction was significant ($P < 0.05$) only in the group that received iron as well as zinc. Diarrhea episodes were reduced by 37% in the zinc group ($P < 0.05$) and by 28% in the zinc + iron group ($P < 0.05$). Although the differences were not significant, there were fewer total episodes of respiratory disease and fewer episodes per child in both groups supplemented with zinc. When data were combined for both groups who received zinc, compared with the other two groups combined, there was a significant reduction in the total number of episodes of disease (3.8 ± 0.3 compared with 5.0 ± 0, 3, $P < 0.01$) and of diarrhea (0.8 ± 0.1 compared with 1.3 ± 0.2, $P < 0.01$), but not in the number of episodes of respiratory illness (2.7 ± 0.2 compared with 3.4 ± 0.3) or other disease. None of the supplements reduced the duration of either diarrheal or respiratory disease or affected the number of episodes of fever. Iron supplementation alone had no effect on any morbidity.

DISCUSSION

Neither zinc nor iron supplementation for 12 mo produced any improvement in the anthropometry of these growth-stunted preschoolers. At baseline the children had a high prevalence of iron deficiency. Iron supplementation produced a significant increase in mean plasma ferritin concentrations and low ferritin values disappeared in the two iron-supplemented groups. There was a spontaneous improvement in hemoglobin in the placebo group, for reasons that are unknown, so that there was no significant effect of the iron supplements on the final hemoglobin concentration. From this and previous studies in these communities (28) it is apparent that hemoglobin synthesis is being limited by multiple micronutrient deficiencies. It is less certain that these children were severely zinc depleted, because their initial mean plasma zinc concentration was > 13.8 μmol/L. However, 20% of them had a baseline value < 10.7 μmol/L. After 12 mo of supplementation mean plasma zinc

TABLE 3
Changes in body-composition indicators between baseline and 12 mo in the four groups[1]

	Placebo	Iron	Zinc	Zinc + iron
All children[2]				
MAC (cm)	0.67 ± 0.08	0.73 ± 0.08	0.93 ± 0.02	0.68 ± 0.07
TST (mm)	0.33 ± 0.27	0.46 ± 0.24	0.59 ± 0.30	0.74 ± 0.27
MAMA (cm^2)	1.00 ± 0.18	1.03 ± 0.15	1.20 ± 0.26	0.78 ± 0.16
MAFA (cm^2)	0.57 ± 0.18	0.63 ± 0.16	0.83 ± 0.21	0.76 ± 0.17
Children with initial HAZ < −2.0[3]				
MAC (cm)	0.73 ± 0.15	1.22 ± 0.11	0.94 ± 0.15	0.83 ± 0.14
TST (mm)	0.31 ± 0.31	0.63 ± 0.53	0.82 ± 0.45	0.63 ± 0.50
MAMA (cm^2)	1.25 ± 0.29	1.79 ± 0.25	1.20 ± 0.21	1.07 ± 0.28
MAFA (cm^2)	0.46 ± 0.26	0.97 ± 0.32	0.97 ± 0.31	0.76 ± 0.32

[1] $\bar{x}$ ± SEM. No means were significantly different from the placebo group. MAC, midarm circumference; TST, triceps skinfold thickness; MAMA, midarm muscle area; MAFA, midarm fat area; HAZ, height-for-age *Z* score.
[2] n = 46 in the placebo, 46 in the iron, 47 in the zinc, and 47 in the zinc + iron group.
[3] n = 19 in the placebo, 17 in the iron, 18 in the zinc, and 17 in the zinc + iron group.

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TABLE 4
Changes in biochemical indicators between baseline and 12 mo of supplementation in the four groups[1]

	Placebo		Iron		Zinc		Zinc + iron	
	0 mo	12 mo	0 mo	12 mo	0 mo	12 mo	0 mo	12 mo
Hemoglobin (g/L)	108 ± 14	118 ± 8	108 ± 13	118 ± 9	109 ± 11	118 ± 7	107 ± 10	118 ± 7
Ferritin (μg/L)	20.1 ± 44.6	22.9 ± 16.5	21.2 ± 38.1	46.9 ± 24.2[2]	18.9 ± 15.8	19.7 ± 13.5	14.7 ± 15.6	43.6 ± 22.9[3]
Plasma zinc (μmol/L)	14.2 ± 0.7	14.4 ± 0.6	15.2 ± 0.6	15.2 ± 0.7	13.2 ± 0.6	16.8 ± 0.8[4]	16.5 ± 0.6	18.3 ± 0.7[4]

[1] $\bar{x}$ ± SEM.
[2–4] Significantly different from initial value; [2] $P < 0.05$, [3] $P < 0.001$, [4] $P < 0.01$.

concentration rose by > 3 μmol/L in the zinc group and by > 1.5 μmol/L in the group supplemented with both zinc and iron. The fact that zinc supplementation reduced morbidity could be taken as evidence that initial zinc status was suboptimal in the study sample as a whole.

From a recent meta-analysis of data from zinc intervention trials designed to improve growth, the predictors of length or height growth response to zinc supplementation were low initial HAZ and low initial plasma zinc concentration, but not age at the time of supplementation (KH Brown, J Pearson, LH Allen, unpublished observations, 1996). Several studies have reported a positive effect of zinc on growth in older, but more severely stunted, children (11). Thus, one possible explanation for lack of growth or body composition response to zinc supplementation in these Mexican children is that they were not severely stunted or zinc deficient. Although there was no significant effect of the supplement on growth of those children with an initial HAZ < −2 or with an initial plasma zinc concentration < 10.7 μmol/L, the lack of significance might be due to the small number of these children in each treatment group. However, the largest difference in height velocity between any of the groups was 0.6 cm among stunted children, which would have needed a sample size of > 300 children per group to be detected with 80% power. Also, a height difference of 0.6 cm over the course of a year is likely to be unimportant from a practical or functional point of view. Likewise, a sample size of 350 initially stunted children per group would have been needed for the triceps-skinfold-thickness differences to be significant.

Based on previous data from Mexico, it was surprising that only 20% of the children had a plasma zinc concentration < 10.7 μmol/L at baseline. We observed the apparent absorption of zinc from similar maize-based, high-phytate Mexican diets to be < 5% when they were fed to well-nourished adults (17). Using food intake data from children of the same age collected during previous research in this community, Murphy et al (29) calculated their mean zinc intake to be 5.3 mg/d, and predicted that 68% of the children had an inadequate zinc intake, after correction for zinc bioavailability. It is possible that zinc status was protected to some extent by adaptations such as reduced endogenous secretion of the mineral (30). Calculated iron intakes for the same age group averaged 6.8 mg/d. The amount of absorbable iron consumed was predicted

TABLE 5
Morbidity of Mexican children during 12 mo of supplementation in the four groups[1]

Morbidity measure	Placebo (n = 56)	Iron (n = 54)	Zinc (n = 54)	Zinc + iron (n = 55)
Total disease episodes (n)	255	285	211	202[1]
Total respiratory	179	192	163	139
Total diarrheal	62	76	40[1]	46[1]
Other	14	17	8	17
Episodes/child (n)				
Total all episodes[2]	4.6 ± 0.5[3]	5.4 ± 0.5	3.9 ± 0.3	3.7 ± 0.4
Respiratory	3.2 ± 0.4	3.6 ± 0.4	3.0 ± 0.3	2.5 ± 0.3
Diarrheal[4]	1.1 ± 0.2	1.4 ± 0.2	0.7 ± 0.1	0.8 ± 0.1
Other	0.3 ± 0.08	0.3 ± 0.04	0.1 ± 0.06	0.3 ± 0.09
Duration of episodes				
Total all episodes (n)[5]	142	162	113	106
Respiratory (d)	9.9 ± 0.5	9.8 ± 0.5	9.4 ± 0.5	9.4 ± 0.6
Diarrheal (d)	5.0 ± 0.6	4.3 ± 0.6	4.4 ± 0.4	6.0 ± 1.0
Other (d)	10.1 ± 1.5	12.5 ± 2.4	11.0 ± 1.6	14.1 ± 2.9
Episodes of fever (n)				
Total all disease episodes[5]	235	276	190	191
Episodes with fever[6]	48 [20]	60 [22]	43 [23]	53 [28]

[1] Significantly different from placebo group, $P < 0.05$.
[2,4] Significantly reduced in the zinc-supplemented groups; [2] $P < 0.035$, [4] $P < 0.01$.
[3] $\bar{x}$ ± SEM.
[5] Number reduced because of missing information on disease duration and fever.
[6] Percentage in brackets.

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to be inadequate to prevent anemia in 43% of the children, or to provide adequate iron stores for 88% of them (29).

Another possible reason for the lack of an effect of zinc on linear growth in our study could be the coexistence of other micronutrient deficiencies that could be limiting growth. At baseline, 64% of these children had deficient plasma α-tocopherol concentrations (< 5 mg/L), 24% had low (< 200 μg/L), and 5% had deficient (< 100 μg/L) plasma retinol concentrations, 33% had low (< 200 ng/L) and 10% had deficient (< 300 ng/L) plasma vitamin B-12 concentrations, and 5% had deficient and 28% had low riboflavin intakes based on the erythrocyte glutathione reductase activity coefficient (unpublished observations). There has been little systematic study of how these or other micronutrient deficiencies might limit the growth of young children (11, 31).

Most studies have reported no evidence for an effect of iron supplementation on the growth of anemic or iron-deficient children (11). Exceptions include the work of Angeles et al (20), who showed a positive effect of iron (30 mg/d) and ascorbic acid (20 mg/d) supplementation on linear growth of anemic Indonesian children compared with a supplement of ascorbic acid alone. The effect was attributed to a reduction in the episodes of respiratory infections and diarrhea observed with iron supplementation. Lawless et al (32) showed a significant increase in appetite and growth when anemic primary schoolchildren in Kenya were supplemented with 30 mg Fe/d for 14 wk. We conclude that iron supplementation did not affect the growth or body composition of these Mexican children, even though there was a high prevalence of iron deficiency before supplementation, the duration of the intervention was long, and the iron deficiency was corrected.

Zinc supplementation reduced the number of morbid episodes in these growth-stunted rural Mexican preschoolers. Specifically, those who received a zinc supplement for 12 mo, either as zinc alone or zinc + iron, had significantly fewer episodes of disease and diarrhea compared with children who received the placebo or iron alone. Zinc is important for the integrity of the immune system (21), and supplements have improved the immunocompetence of malnourished children (33, 34). Both severe and mild zinc deficiency can contribute to the duration and severity of existing diarrheal disease (35, 36). In addition, zinc supplementation of infants with acute and persistent diarrhea was shown in one study to improve mucosal integrity (37). Future studies should investigate the mechanism by which zinc reduces the number of morbid episodes. Diarrhea has been shown in other studies to be associated with stunting, malabsorption and excessive excretion of nutrients, and childhood mortality. Thus, if the results of the present study are confirmed, improving the zinc status of growth-stunted, marginally zinc-deficient children might prove to be an important public health strategy. Because providing iron in addition to zinc did not affect growth or morbidity in this study, and iron and zinc deficiency are likely to occur simultaneously in populations whose diets are high in phytate and/or low in animal products, it is logical to provide iron as well as zinc supplements in regions where iron deficiency is endemic.

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The nsp15 Nuclease as a Good Target to Combat SARS-CoV-2: Mechanism of Action and Its Inactivation with FDA-Approved Drugs

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Abstract: The pandemic caused by SARS-CoV-2 is not over yet, despite all the efforts from the scientific community. Vaccination is a crucial weapon to fight this virus; however, we still urge the development of antivirals to reduce the severity and progression of the COVID-19 disease. For that, a deep understanding of the mechanisms involved in viral replication is necessary. nsp15 is an endoribonuclease critical for the degradation of viral polyuridine sequences that activate host immune sensors. This enzyme is known as one of the major interferon antagonists from SARS-CoV-2. In this work, a biochemical characterization of SARS-CoV-2 nsp15 was performed. We saw that nsp15 is active as a hexamer, and zinc can block its activity. The role of conserved residues from SARS-CoV-2 nsp15 was investigated, and N164 was found to be important for protein hexamerization and to contribute to the specificity to degrade uridines. Several chemical groups that impact the activity of this ribonuclease were also identified. Additionally, FDA-approved drugs with the capacity to inhibit the in vitro activity of nsp15 are reported in this work. This study is of utmost importance by adding highly valuable information that can be used for the development and rational design of therapeutic strategies.

Keywords: SARS-CoV-2; ribonucleases; nsp15; inhibitors; FDA-approved compounds; therapeutics

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Citation: Saramago, M.; Costa, V.G.; Souza, C.S.; Bárria, C.; Domingues, S.; Viegas, S.C.; Lousa, D.; Soares, C.M.; Arraiano, C.M.; Matos, R.G. The nsp15 Nuclease as a Good Target to Combat SARS-CoV-2: Mechanism of Action and Its Inactivation with FDA-Approved Drugs. *Microorganisms* **2022**, *10*, 342. https://doi.org/10.3390/microorganisms10020342

Academic Editor: Grzegorz Wegrzyn

Received: 16 December 2021
Accepted: 20 January 2022
Published: 1 February 2022

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1. Introduction

Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) emerged in 2019 in Wuhan, China [1] and rapidly spread worldwide, initiating a global pandemic. This virus has caused a major impact on health systems and in the world economy. In the last two years, scientists all around the world have extensively worked to better understand how this virus functions. A profound knowledge of the mechanisms involved in viral replication is of the utmost urgency and will surely contribute to the development of new treatments against COVID-19, the disease caused by SARS-CoV-2.

SARS-CoV-2 is a (+)-sense single-stranded RNA virus with a genomic RNA (gRNA) of ~30 kb in length [2] from the *Nidovirales* order. The gRNA, similar to what is observed in human RNA molecules, has a cap at the 5′-end and a poly(A) tail at the 3′-end. The presence of these traits allows the immediate translation of the viral genome in order to produce viral proteins, and prevent its degradation by intracellular ribonucleases (RNases) [3,4]. SARS-CoV-2 replication is a complex and crucial process. It involves the concerted action of viral and host proteins engaged in RNA polymerization, proofreading and capping of the RNA molecule essential in producing new viral proteins [5]. As an RNA virus, it requires a

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tight control on the production and processing of the RNA molecules. Ribonucleases are key proteins in this process [6]. The SARS-CoV-2 genome contains two large open reading frames (ORF), ORF1a and ORF1ab, with the latest coding for nonstructural proteins (nsp). Among the nsp proteins, we can find two ribonucleases, the exoribonuclease nsp14 and the endoribonuclease nsp15 [3]. Nsp14 has recently been characterized [7].

nsp15 is a conserved uridine-specific endoribonuclease only found in viruses from the order *Nidovirales* [8,9]. Considering that no homologs are present in any other RNA viruses, nsp15 is considered a genetic marker of this order of viruses [8,9]. Only distant homologs (XendoU) were identified in some prokaryotes and eukaryotes, which were shown to be involved in the processing of intron-encoded small nucleolar RNAs [10,11]. Nsp15 is a member of the nidoviral EndoU (also known as NendoU) family of proteins. It specifically cleaves RNA substrates endonucleolytically at the 3′ of pyrimidines, with a preference for uridine bases, releasing products with 2′ to 3′ cyclic phosphate and 5′-hydroxyl ends [12]. Nsp15 activity can be influenced by the secondary structure of the RNA, and has a preference for cleaving unpaired uridine bases in hairpin-structured RNAs [12]. It was also reported that the presence of 2′-*O*-ribose methyl groups on the RNA substrate can block the nsp15 activity [8]. Downstream to nsp15, the *nsp16* gene encodes for 2′-*O*-ribose methyltransferase [3]. Taking this into account, we can hypothesize that nsp16 may be a regulator of nsp15 activity by avoiding the cleavage of important viral RNA molecules during the infection process.

Biochemical studies performed with purified nsp15 from SARS-CoV and HCoV-229E revealed that this ribonuclease requires divalent ions as a cofactor to perform RNA cleavage, with a marked preference for Mn^{2+} [8,13]. These studies also revealed that Mn^{2+} influences nsp15 conformation, enhances RNA binding and, consequently, its NendoU activity [8,13]. Structural studies with nsp15 from different coronaviruses revealed that this protein is a hexamer formed by dimers of trimers, with a distinctive folding from other ribonucleases (Figure 1A) [14–19]. Each monomer is composed of three domains: N-terminal domain (ND), middle domain (MD) and the C-terminal catalytic NendoU domain (Figure 1B,C) [14–19]. The NDs are lined in the center of the hexamer, while the NendoU domains are facing the surface, which means that nsp15 has six active sites (Figure 1C) [14–19]. The stabilization of the hexamer is achieved through interactions of the ND, which contains the residues required for oligomerization, but the other domains also contribute to the oligomer interface [18,20]. The hexamer of nsp15 is the active form that exhibits the ability to bind RNA and the endonucleolytic activity [20]. The catalytic site at the NendoU domain is composed of the highly conserved residues H235, H250 and K290 (numbering according to the SARS-CoV-2 PDB 6VWW [17]) (Figure 1D) that constitute the catalytic triad, similar to what was described for bovine RNase A [21]. This suggests a similar mechanism of action, but while RNase A does not require a cofactor, nsp15 activity is stimulated by Mn^{2+} [12]. S294 and Y343 residues were postulated to interact with the substrate and confer a specificity for uridines [16]. The highly conserved T341 can also be involved in substrate recognition [14].

nsp15 plays an important role in coronavirus pathogenesis. It was reported that this RNase is responsible for the degradation of viral dsRNA intermediates, thus preventing host recognition [22,23]. By obstructing the recognition of the viral RNA, nsp15 directly contributes to delaying the type I interferon response. Several studies using live viruses in primary cells have confirmed that nsp15 acts as an interferon antagonist [22,23]. Later on, it was described that coronaviruses with mutations in the NendoU domain are less virulent, and their ability to activate the innate immune response is compromised [24]. For a review on this topic, please read Reference [25]. Taking all this into consideration, plus the fact that nsp15 is only found in nidoviruses with no close human homologs identified, this endoribonuclease is a very attractive target for developing new drugs to treat SARS-CoV-2 infections.

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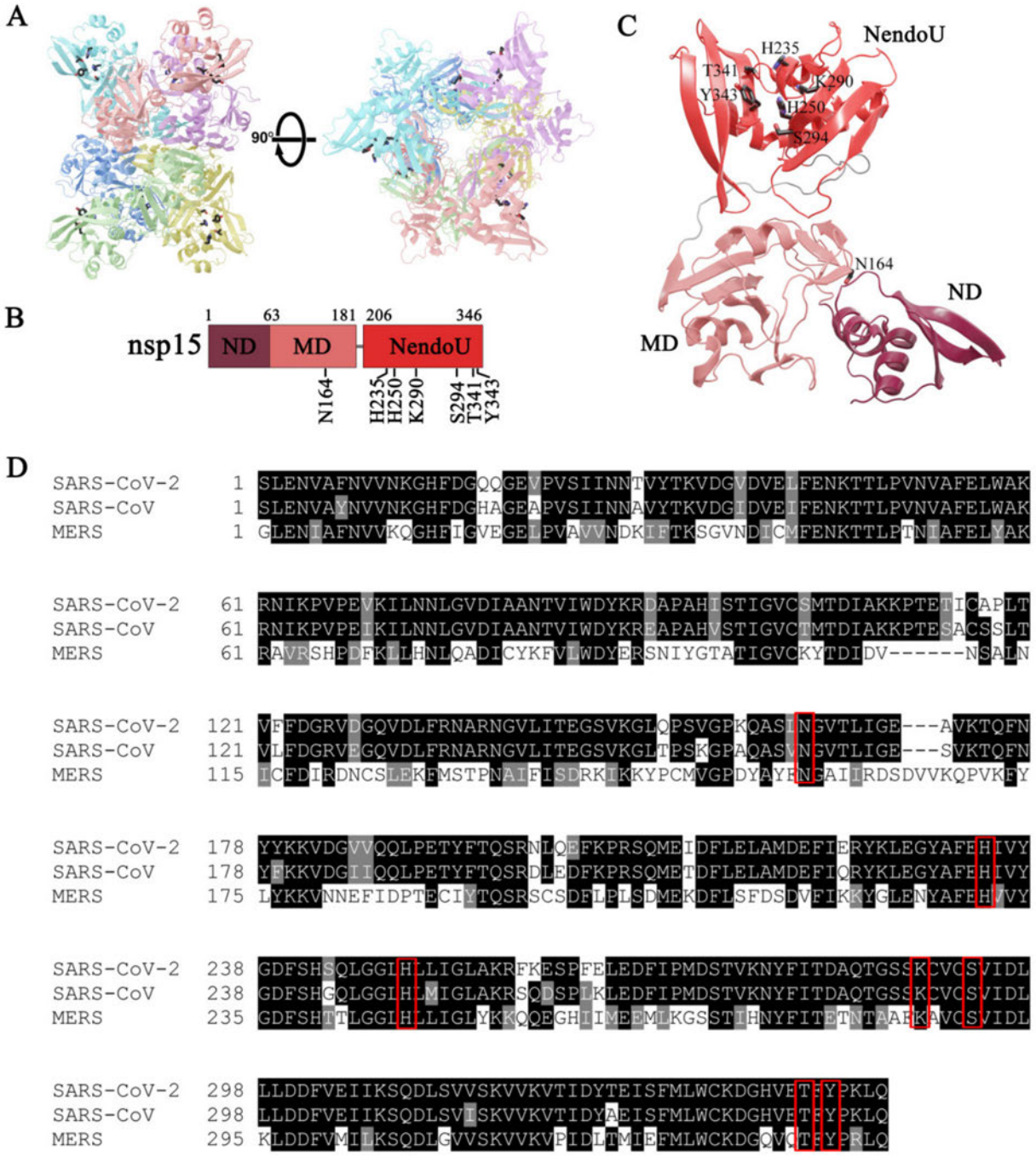


Figure 1. (**A**) Orthogonal views of the hexameric form of nsp15, which is formed by dimers of trimers. Monomers are colored in cyan, pink, violet, blue, green and yellow. Active site residues are shown as sticks. (**B**) nsp15 domain organization. ND, amino acids (aa) 1–63; MD, aa 64–181; NendoU, aa 206–346. The location of the conserved residues is represented. (**C**) Localization of the nsp15 domains in the monomer (PDB code 6W01). Mutated residues are shown as sticks. (**D**) Sequence alignment of nsp15 from SARS-CoV-2 (UniProt ID: P0DTD1), SARS-CoV (UniProt ID: P0C6X7) and MERS-CoV (YP_009047225). Residues are colored according to their conservation: in black, residues that are identical; in grey, residues that are not identical but have similar properties; in white, residues that are different. Residues mutated in this work are highlighted with a red box.

In this work, we elucidated the mechanism of action of nsp15 from SARS-CoV-2. Based on this knowledge, we also performed an *in silico* screening to find the best commercially available and approved drugs that might inhibit the NendoU activity of nsp15.

FOI 26-3132 - Document 5

2. Materials and Methods

2.1. Plasmid Construction

Full-length nsp15 gene from SARS-CoV-2 (UniProt ID P0DTD1) was optimized for *Escherichia coli* expression and synthesized by GenScript (Piscataway, NJ, USA). The synthesized gene was subsequently cloned into the *Nde*I–*Bam*HI sites of commercial pET15b to generate pET15b_nsp15, which expresses an N-terminal His-tagged version of nsp15.

The single-point mutations N164A, H235A, H250A, K290A, S294A, T341A and Y343A and the double mutant T341A_Y342A were introduced into pET15b_nsp15 by overlapping PCR using the primers listed in Table S1. The double mutants S294A_T341A and S294_Y343A and the triple mutant S294A_T341A-Y343A were introduced into pET15b_nsp15_S294A also by overlapping PCR (Table S1). All the constructions were verified by sequencing at StabVida (Caparica, Portugal).

2.2. Protein Expression and Purification

Plasmids expressing nsp15 WT and mutant proteins were transformed into *E. coli* Rosetta cells (Novagen, Gibbstown, NJ, USA) for the expression of the recombinant proteins. Cells were grown in Terrific Broth medium supplemented with 150 μg/mL ampicillin and 50-μg/mL chloramphenicol at 30 °C to an OD_{600} of 0.5. At this point, protein expression was induced by addition of 0.5 mM IPTG, and bacteria were incubated for 4 h. Cells were pelleted by centrifugation and stored at −80 °C. Cell suspensions were lysed using the FastPrep-24 (MP Biomedical, Santa Ana, California, USA) at 6.5 m/s for 60 s in the presence of 0.5-mM PMSF. The crude extract was treated with Benzonase (Sigma-Aldrich, St. Louis, MO, USA) to degrade the nucleic acids, and clarified by a 30-min centrifugation at 10,000× *g*. Purification was performed in an ÄKTA FPLC™ system (Cytiva, Marlborough, MA, USA). The cleared lysate was subjected to a histidine affinity chromatography in a HisTrap HP column (Cytiva) equilibrated in Buffer A (40-mM Tris-HCl, pH 8, 150-mM NaCl and 10-mM imidazole). Proteins were eluted by a continuous imidazole gradient up to 500 mM in Buffer A. The fractions containing the purified protein were pooled together and concentrated by centrifugation at 4 °C with Amicon Ultra Centrifugal Filter Devices of 10000 MWCO (Millipore, Burlington, Massachusetts, EUA) and the buffer exchanged to Buffer B (20-mM Tris-HCl, pH 8). The proteins were then added to a HiTrap Q HP column (Cytiva) equilibrated in Buffer B to perform an anion exchange. Protein elution was achieved by a continuous NaCl gradient up to 1 M in Buffer B. Eluted proteins were concentrated by centrifugation at 4 °C with Amicon Ultra Centrifugal Filter Devices of 10000 MWCO (Millipore) and the buffer exchanged to Buffer C (20-mM Tris-HCl, pH 8 and 150-mM NaCl). Afterwards, the proteins were subjected to a size exclusion chromatography using a Superdex 200 Increase column (Cytiva) with a flow rate of 0.5 mL/min using buffer C. The samples collected were analyzed in a Novex™ 8–16% Tris-Glycine gel (Invitrogen™, Waltham, Massachusetts, EUA), followed by BlueSafe staining (NZYTech, Lisbon, Portugal). Samples with the highest purity were pooled together and concentrated by centrifugation at 4 °C with Amicon Ultra Centrifugal Filter Devices of 10000 MWCO (Millipore). All protein versions were purified at least twice in independent experiments to ensure reproducibility of the results. Proteins were quantified using the Bradford method, and 50% (*v/v*) glycerol was added to the final fractions prior storage at −20 °C. The purified proteins were analyzed by Western blot. One and a half micrograms of each protein was separated in a Novex™ 8–16% Tris-Glycine gel (Invitrogen™) and transferred to a nitrocellulose membrane (Hybond ECL, Cytiva) by electroblotting using the Trans-Blot SD semidry electrophoretic system (Bio-Rad, Hercules, CA, USA). Membranes were probed with a 1:10,000 dilution of anti-His antibody (Thermo Fisher Scientific, Waltham, MA, USA). ECL anti-mouse IgG conjugated horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was used as the secondary reagent in a 1:10,000 dilution. Immunodetection was conducted via a chemiluminescence reaction using Amersham ECL Western Blotting Detection Reagents (Cytiva).

FOI 26-3132 - Document 5

2.3. RNase Activity Assays

The synthetic 16-mer oligoribonucleotides R16.4 (5′-GAAGCGAAACCCUAAG-3′), 16comp (5′-AGUGGUUGGUGUCGGG-3′) and 30-mer (5′-CCCGACACCAACCACUAAAAAAAAAAAAAA-3′) (StabVida, Portugal) were used as substrates in the RNase activity assays. The RNA substrates were labeled at their 5′ end with [^{32}P]-γ-ATP and T4 Polynucleotide Kinase (Ambion, Austin, TX, USA) in a standard reaction. MicroSpin G-50 columns (Cytiva) were used to remove excess [^{32}P]-γ-ATP. In order to fold the R16.4 into its native structure, the RNA was resuspended in 10 mM of Tris-HCl, pH 8.0 and incubated 10 min at 80 °C, followed by 45 min at 37 °C. To obtain the double-stranded substrate 16–30 ds, the labeled 30-mer was hybridized to the unlabeled 16comp oligoribonucleotide. The hybridization was performed in a 1:5 (mol:mol) ratio by incubation for 10 min at 80 °C, followed by 45 min at 37 °C.

The activity assays were performed in a final volume of 12 µL containing an activity buffer with 25-mM HEPES, pH 7.4; 50-mM NaCl; 1-mM DTT; 10-mM $NaHPO_4$ and 5 mM of either $MgCl_2$, $MnCl_2$, $CaCl_2$, $NiCl_2$, $CuCl_2$, $CoCl_2$ or $ZnCl_2$, plus the protein nsp15 WT or respective mutants (protein concentrations are indicated in the figure legends). Some assays were performed with different concentrations of $MnCl_2$ (1 mM, 5 mM, 10 mM, 15 mM or 20 mM) or with an activity buffer containing citrate or ammonium sulphate instead of sodium phosphate. A reaction was also done only in a HEPES buffer, without citrate, sulphate or $NaHPO_4$ (as indicated in the figure legends). All the reactions were started by adding 50 nM of the RNA substrate and further incubated at 37 °C. Reaction controls were performed with all the components of the reaction without the enzymes. They were incubated in the same conditions as in the enzymatic reactions.

To test the drugs (Dutasteride, Etoposide, Golvatinib, Irinotecan, Meprednisone and Tasosartan), the compounds were previously incubated for 7 min at room temperature with nsp15 WT before the reactions were started (through the addition of RNA). All the drug compounds were solubilized in DMSO and serially diluted. Reaction controls were performed with the RNA substrates (without the enzymes), with either the activity buffer or buffer supplemented with DMSO. The DMSO concentration was kept constant, corresponding to the maximum amount of drugs tested in the assay. The control reactions were incubated in the same conditions.

For all the assays, aliquots of 4 µL were withdrawn at the time points indicated in the respective figures, and the reactions were stopped by adding a gel-loading buffer containing 80% *(v/v)* formamide and 10-mM EDTA. Reaction products were resolved in a 20% denaturant polyacrylamide gel (7-M urea). Signals were visualized by PhosphorImaging (TLA-5100 Series, Fuji, Cytiva). All the experiments were performed at least in triplicate for each independent purification. Disappearance of the substrate over time was quantified using ImageQuant software (Cytiva).

2.4. Sequence Alignment

nsp15 sequences from SARS-CoV-2 (UniProt ID: P0DTD1), SARS-CoV (UniProt ID: P0C6X7) and MERS-CoV (YP_009047226) were aligned using T-coffee [26]. The final figure presented was built with Boxshade (https://embnet.vital-it.ch/software/BOX_form.html, accessed on 3 September 2021). Sequence identity and similarity were determined in https://www.bioinformatics.org/sms2/ident_sim.html, accessed on 3 September 2021).

2.5. Docking Calculations

The tridimensional structures of the target molecules were obtained from the Approved, Investigational and Experimental subsets of the Drugbank database [27]. All molecules were prepared using AutoDockTools [28] by adding Gasteiger charges and AutoDock4 atom types. Flexible bonds were allowed to rotate, nonpolar hydrogens were merged and missing polar hydrogens were added. The structure present in PDB ID 6WXC was chosen to be the receptor, since this protein was co-crystalized with a drug and a phosphate ion bound together to the active site. The protein was prepared with AutoDock-

FOI 26-3132 - Document 5

Tools in a similar way as the drugs, and all atoms that were not part of the protein or the phosphate ion were removed. Docking calculations were performed with the program AutoDock Vina [29] in a searching box with sizes 22 Å, 24 Å and 20 Å (*x*-, *y*- and *z*-axis, respectively) centered at the nsp15 active site. The exhaustiveness parameter was set to 20 for all runs, and only the poses with the lowest binding energies were considered for further analysis.

3. Results

3.1. nsp15 Endoribonuclease Is Active as a Hexamer

We purified the SARS-CoV-2 nsp15 recombinant protein. As presented in Figure 2A, the chromatogram obtained during the size exclusion chromatography step shows the presence of three main protein peaks that elute between 9 and 16 mL of column volume. It was already demonstrated that nsp15 is a hexamer formed by dimers of trimers [14–19]. As such, and taking into consideration the chromatograms with standard components, we postulate that the first peak corresponds to the hexamer (H), the second and smaller peak to the trimer (T) and the last one to the monomer (M) (Figure 2A). The fractions collected for each peak were analyzed in an SDS-PAGE gel. We confirmed the presence of a protein that migrates at 41 kDa (Figure 2A), which is consistent with the expected molecular weight of the recombinant His-tagged nsp15. It is possible to observe bands with higher molecular weight in the gel, namely in the fractions that correspond to the hexameric and trimeric forms. By Western blot, using antibodies specific for the His-tag tail of the protein, we confirmed that those bands correspond to nsp15 oligomers, which are not present in the fraction of the monomeric form (Figure 2A). We could also observe the presence of bands with lower molecular weights in the trimer and monomer fractions (Figure 2A). These bands most likely corresponded to nsp15 that was degraded during the purification process.

We tested the endonucleolytic activity of the purified fractions, using the synthetic RNA substrate R16.4, previously reported in Bhardwaj et al. [12]. This substrate is a 16-nucleotide (nt)-long RNA that contains only one uridine, rendering an unique cleavage event by nsp15 [12]. The results in Figure 2B show that nsp15 is more active as a hexamer. It retains some activity in the trimeric form, and only residual cleavage is observed in the monomeric form (Figure 2B). These results are in agreement with previous studies [20]. Moreover, it is possible to observe that the incubation of SARS-CoV-2 nsp15 with the R16.4 RNA substrate originates a single degradation product, due to cleavage on the U residue (Figure 2B), which demonstrates the preference of this enzyme for uridines. We also tested the activity of nsp15 over the ssRNA 16comp that presents five uridines in its sequence (Figure 2C). As expected, it is possible to observe the generation of five degradation products that correspond to the cleavage at the uridines (Figure 2C, left panel), reinforcing the marked preference of nsp15 for U residues. It was recently described that nsp15 has a preference for purines at the 3′ position of the cleaved uridines [30]. Interestingly, the 16comp RNA has purines in the 3′ position of the first four uridines (U3, U6, U7 and U10) counting from the 5′ end, whereas the last uridine (U12) is followed by a pyrimidine residue (Figure 2C). Analyzing the degradation pattern obtained, we can observe that cleavage is much less efficient at U12 (Figure 2C, left panel), confirming that SARS-CoV-2 nsp15 prefers purines 3′ to uridines.

It was already reported that nsp15 can cleave both ssRNA and dsRNA [8,13]. Therefore, we also tested SARS-CoV-2 nsp15 activity using the 16–30 ds RNA molecule. Our results show that this endoribonuclease is able to cleave this substrate (Figure 2C, right panel), confirming that SARS-CoV-2 nsp15 can cleave both ss and dsRNA molecules.

FOI 26-3132 - Document 5

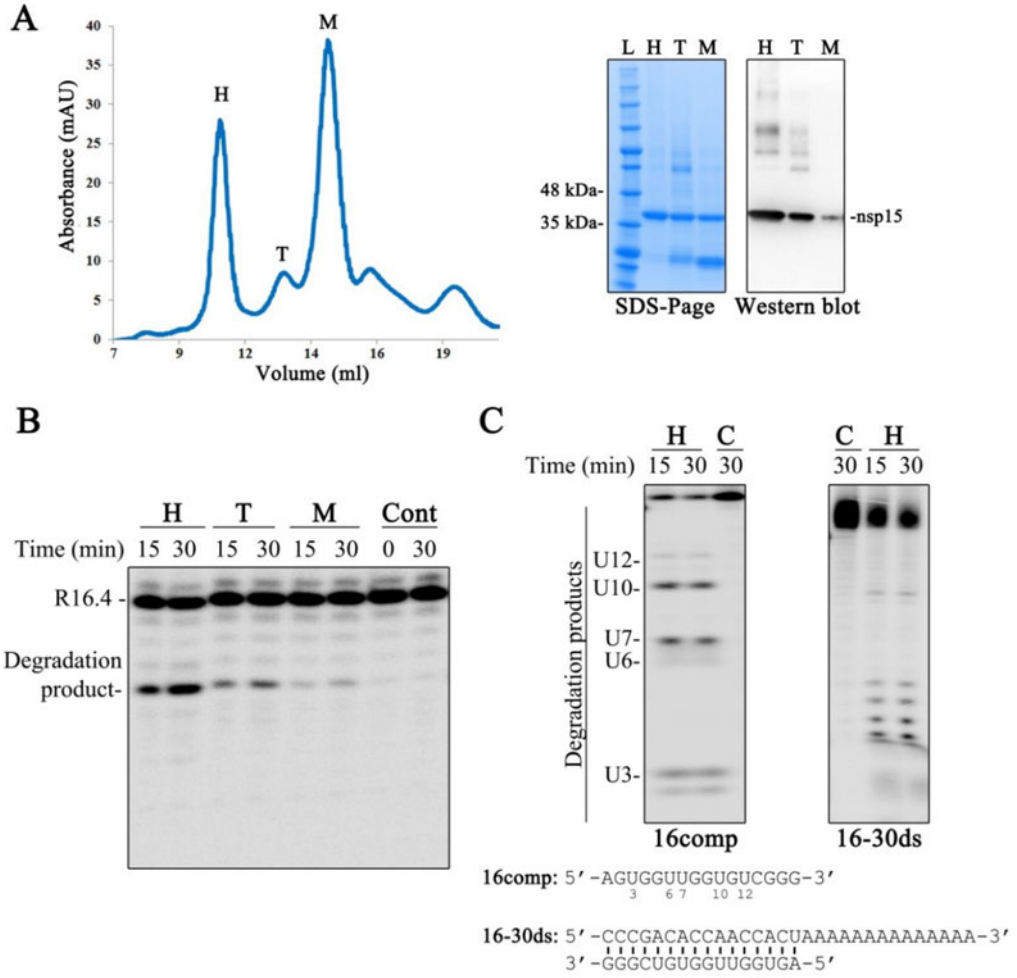


Figure 2. Characterization of SARS-CoV-2 nsp15. (**A**) Left panel: chromatogram obtained during nsp15 size exclusion chromatography; right panels: SDS-PAGE gel with the purified fractions collected during the SEC and the respective Western blot. M, monomer; T, trimer; H, hexamer; L, Protein ladder. (**B**) In vitro activity of the purified fractions H, T and M using 50 nM of R16.4 RNA substrate and 500 nM of protein. (**C**) In vitro activity of nsp15 in the hexamer conformation (500 nM) in the presence of a single-stranded RNA (16comp) and a double-stranded RNA (16–30 ds), which are schematically represented in the bottom. Reactions were analyzed on 7-M urea/20% polyacrylamide gels. C, control reactions; time points are indicated in the top of each panel. All the experiments were performed at least in triplicate.

3.2. nsp15 Activity Is Stimulated in Different Buffer Conditions, with a Marked Preference for Manganese

It was already reported that the activity of nsp15 is highly enhanced in the presence of manganese [13,15,16]. In order to assess the role of other divalent ions over SARS-CoV-2 nsp15 activity, we tested the activity of the enzyme in the presence of Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} (Figure 3A). Our results clearly illustrated that SARS-CoV-2 nsp15 presents maximum activity in the presence of Mn^{2+} (Figure 3A,C). Interestingly, Ca^{2+} and Cu^{2+} can also stimulate nsp15 activity. In the presence of Ni^{2+} and Co^{2+}, the enzyme retains residual activity, while Zn^{2+} does not influence the activity of this RNase (Figure 3A,C). No degradation was observed in presence of the chelating agent EDTA in these conditions. We also tested if the presence of Mn^{2+} or Mg^{2+} would influence nsp15 oligomerization, which would help explain the increase in nsp15 activity. However, the chromatograms were similar to the one obtained in the absence of divalent ions that is presented in Figure 2A. Altogether, these results confirm the importance of divalent ions, namely Mn^{2+}, for the activity of nsp15 from SARS-CoV-2, similar to what was described

FOI 26-3132 - Document 5

for other counterparts [13,15,16], and exclude the involvement of divalent ions in oligomerization. Studies performed with MERS-CoV and SARS-CoV nsp15 have shown that the presence of Mn^{2+}, but not Mg^{2+}, increases the affinity to the RNA molecule [12,16]. This suggests that Mn^{2+} stimulates nsp15 activity by increasing the ability to bind to RNA substrates and not by binding to its active site. In fact, nsp15 structure suggests that divalent ions may stabilize the protein [18,31]. Next, we tested the nsp15 activity using different Mn^{2+} concentrations, from 1 to 20 mM. The results obtained showed that lower Mn^{2+} concentrations are more effective in stimulating nsp15 activity, with the protein presenting a higher ability to cleave RNA in the presence of 1 mM of Mn^{2+} (Figure 3B,C).

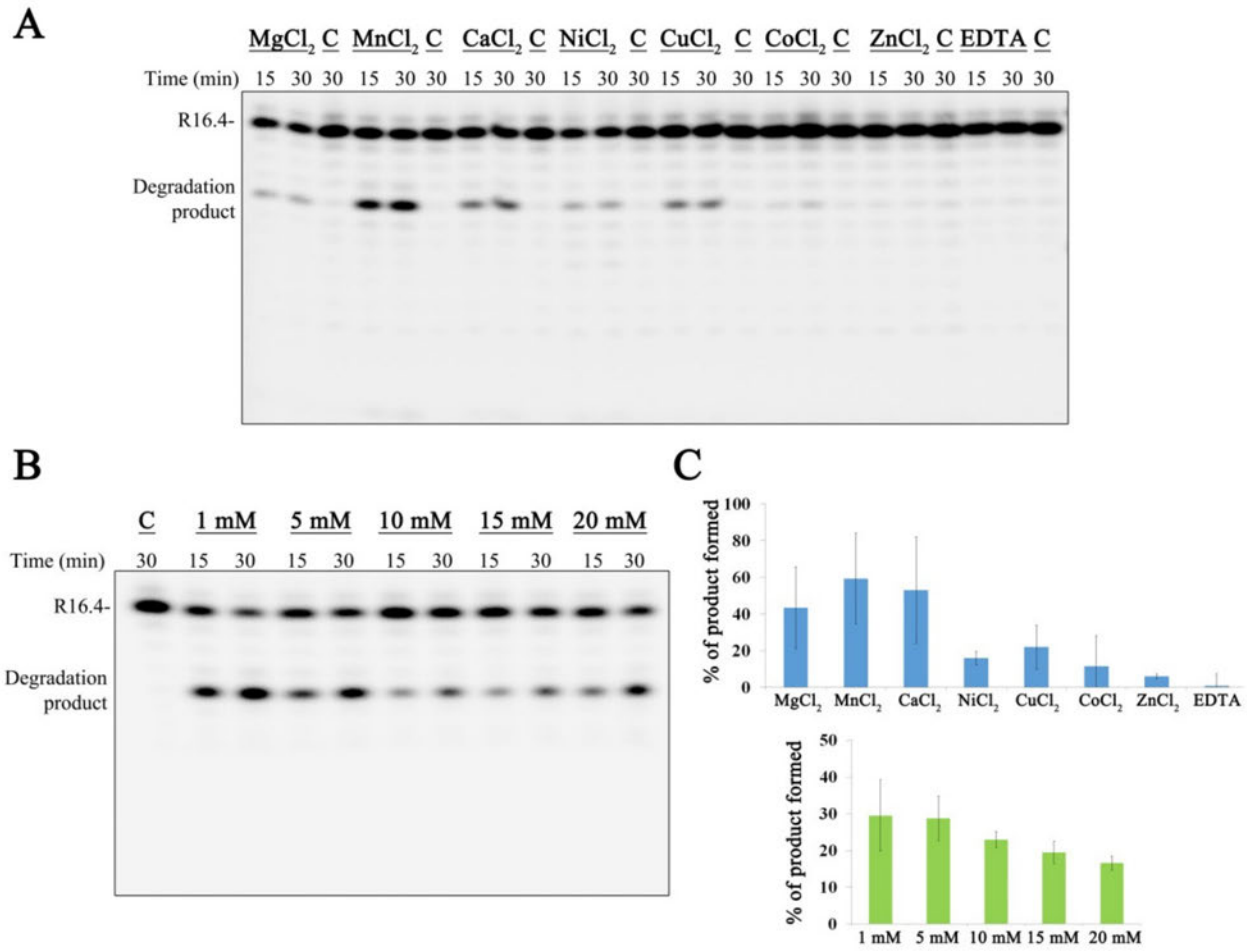


Figure 3. Effect of divalent ions in SARS-CoV-2 nsp15. (**A**) Five hundred nM of nsp15 were incubated with 50 nM of R16.4 RNA substrate in the presence of 10 mM of different divalent ions. (**B**) Five hundred nM of nsp15 were incubated with 50 nM of R16.4 RNA substrate in the presence of different $MnCl_2$ concentrations (1–20 mM). Reactions were analyzed on 7 M urea/20% polyacrylamide gels. C, control reactions; time points are indicated in the top of each panel. All the experiments were performed at least in triplicate. (**C**) Quantification of nsp15 protein activity (% of RNA degradation product generated) in the presence of different divalent ions (on the top) and in a growing range of different $MnCl_2$ concentrations (on the bottom).

Kim et al. reported that nsp15 was crystallized in the presence of a citrate, which was bound to the protein active site [18]. The citrate was shown to make hydrogen bonds with several key and highly conserved residues found in the nsp15 active site, including H235, H250, K290 and T341 (highlighted in Figure 1D). Moreover, it also forms Van der Waals contacts with Tyr343. All these interactions were postulated to stabilize the residues from the active site, thus ordering the NendoU domain [18]. In another study where the nsp15 structure bound to nucleotides was solved, it was possible to observe the presence of

FOI 26-3132 - Document 5

a phosphate ion bound to the active site that also interacted with H235, H250, T341 and K290 residues [31]. It was described that the nsp15 active site is structurally similar to that of RNase A. Sulfate was found to inhibit the ribonucleolytic activity of RNase A [32]. Considering the similarities between both ribonucleases, sulfate could also influence nsp15 activity. In order to determine the role of citrate, phosphate and sulfate over nsp15 activity, we ran activity assays in the presence of these compounds. Our results showed that, in the conditions tested, both phosphate and citrate enhanced nsp15 activity (Figure 4). In contrast, in the presence of sulfate, nsp15 activity was completely blocked (Figure 4). These results indicate that the activity of nsp15 from SARS-CoV-2 is highly influenced by the presence of different chemical groups in the reaction, which can be valuable information when designing compounds to inhibit nsp15 activity.

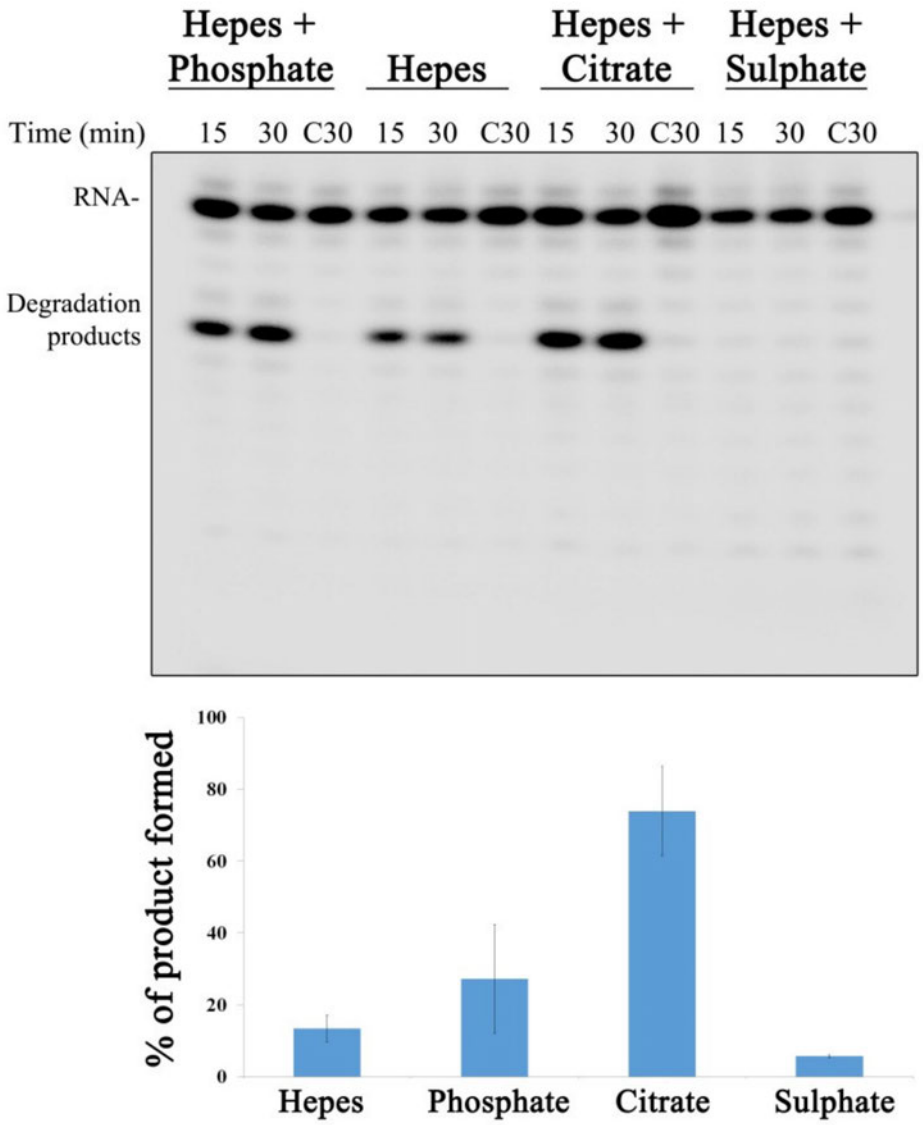


Figure 4. The influence of different buffer compositions in SARS-CoV-2 nsp15 activity. On the top, 500 nM of nsp15 were incubated with 50 nM of R16.4 RNA substrate in the presence of buffers with different compositions, as indicated on the top of each panel. Reactions were analyzed on 7 M urea/20% polyacrylamide gels. C, control reactions; time points are indicated in the top of each panel. All the experiments were performed at least in triplicate. On the bottom, quantification of nsp15 protein activity (% of RNA degradation product generated) in the presence of buffers with different compositions.

FOI 26-3132 - Document 5

3.3. A Residue from ND from SARS-CoV-2 nsp15 Is Important for Hexamer Formation While Residues in NendoU Are Crucial for the Activity of the Protein

nsp15 from different coronaviruses presents a high degree of sequence identity and similarity. SARS-CoV-2 nsp15 shares 88.7% sequence identity and 94.8% of similarity with the SARS-CoV homolog, and 50.1% sequence identity and 63.9% similarity with the homolog from MERS-CoV. The nsp15 active site is constituted by three highly conserved residues (H235, H250 and K290) (Figure 1D) that were postulated to be important for the activity of the enzyme [14,16,18]. Moreover, residues S294, T341 and Y343 were described as important for uridine specificity [16,24,33]. Studies conducted with nsp15 from MERS-CoV have shown that amino acids from the N-terminal portion of the protein (N38, Y58 and N157 in MERS-CoV nsp15 protein) are crucial for hexamer formation [16]. From these, N164 (corresponding to N157 in MERS-CoV) is the only one found in SARS-CoV-2 nsp15 (Figure 1D). Considering the conservation of these amino acids among the three coronaviruses (Figure 1D), and in order to address their role in the activity of SARS-CoV-2 nsp15, we constructed the N164A, H235A, H250A, K290A, S294A, T341A and Y343A point mutants; the S294A_T341A, S294_Y343A and T341A_Y342A double mutants, and the triple mutant S294A_T341A_Y343A. All the mutants were expressed and purified in the same conditions as the wild-type. Size exclusion chromatography showed that the majority of the purified mutants had an elution profile similar to that of the wild-type nsp15, with most of the proteins in hexameric and monomeric forms (Supplementary Figure S1). Interestingly, most of the N164A mutant protein was found in a monomeric form, and only a small peak was detected in the hexameric form (Figure 5A, middle panel). This result showed that N164 residue, which is located in the interface between the N-terminal and NendoU portions of adjacent monomers of nsp15, is crucial for the formation of stable hexamers. This is in agreement with studies performed with MERS-CoV nsp15, where the corresponding residue was also shown to be critical for the formation of the hexamer [16]. In contrast, the K290A mutant presents most of the proteins in the hexameric form (Figure 5A, right panel). This indicates that this residue, which is in the interface between monomers but whose side chain is located in the active site of nsp15, plays a role in the formation of the hexamer. The same behavior was already described for the corresponding residue in SARS-CoV [20]. Taking into consideration that the hexamer is the active form, we analyzed the activity of the fractions corresponding to the hexameric form of all the mutants. For this, we concentrated the samples of each peak corresponding to the hexamer (in order to have enough protein for the activity assays, especially for the N164A mutant) and used two different RNA substrates, R16.4 and the 16comp. As already described above, SARS-CoV-2 nsp15 only cleaves the R16.4 substrate in one place, rendering it a single degradation product (Figure 2B). In contrast, the 16comp substrate has five uridines in its composition, which originates five different degradation products (Figure 2C). We observed that the nsp15 N164A mutant degrades both RNA substrates near the 3′ end and not at the uridine bases as the wild-type protein (Figure 5B,C). This may suggest that mutations in the N-terminal region of the protein may affect its overall folding, compromising the integrity of the active site (NendoU domain). A folding effect is further supported by the unlikelihood of N164 being part of the important interactions between monomers. In fact, the only favorable interaction between N164 and the adjacent monomers are hydrogen bonds with T282. Furthermore, the mutation N164A would represent a decrease of less than 5% in the total contact surface area between monomers. Taking this into account, it is more likely that the predominance of the monomeric form was caused by folding problems and not by the direct absence of hexamer-stabilizing interactions. Although the formation of the hexamer has been severely compromised, N164A still retains a similar activity to the wild-type in terms of substrate consumption (Figure 5B).

FOI 26-3132 - Document 5

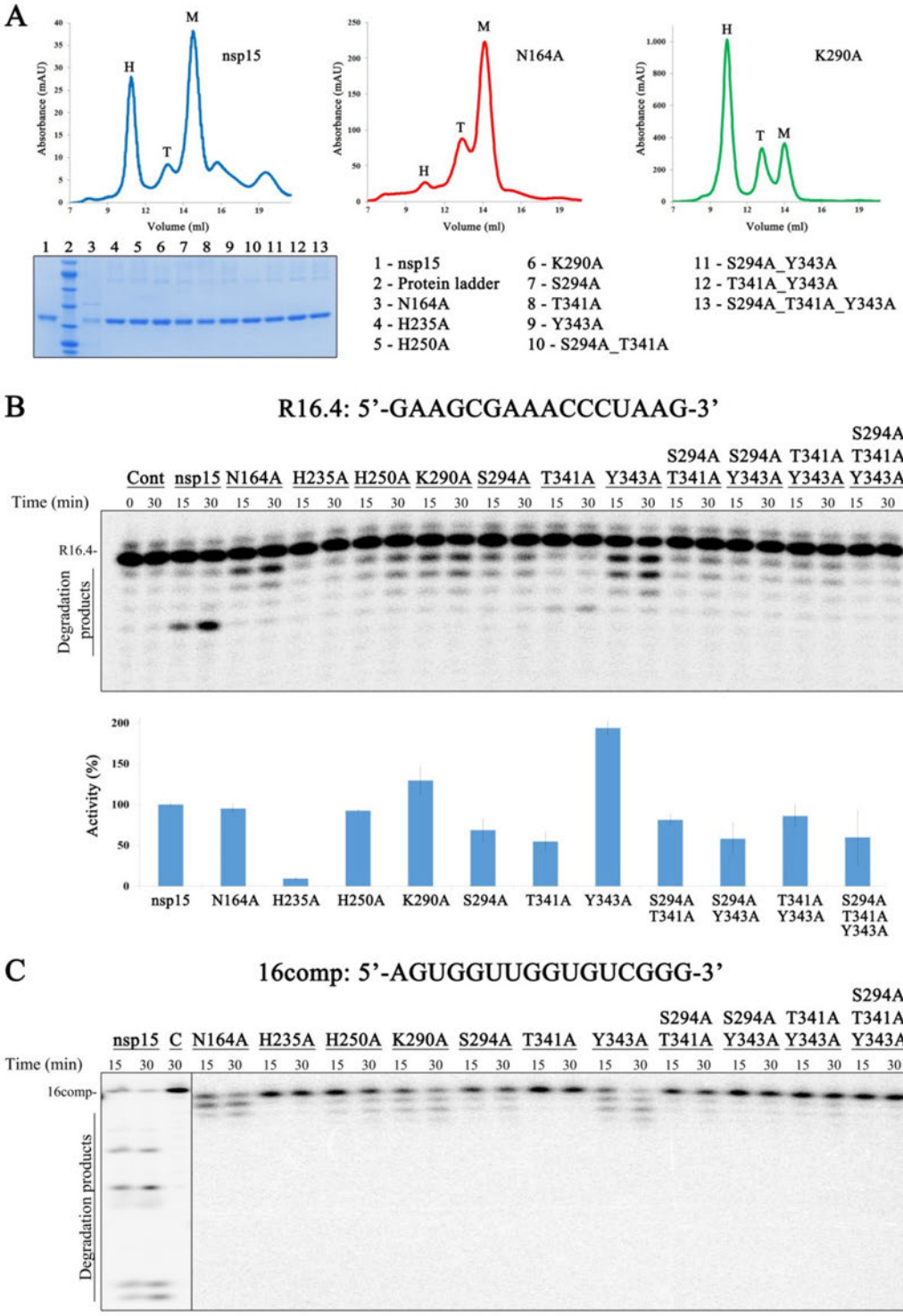


Figure 5. Effect of nsp15 mutations on its endonucleolytic activity. (**A**) On the top, chromatograms obtained during nsp15 wt, N164A and K290A size exclusion chromatographies; M, monomer; T, trimer; H, hexamer; on the bottom, a SDS-PAGE gel with all hexameric fractions of the purified proteins. (**B**) On the top, 800 nM of either nsp15 wt or nsp15 mutant versions were incubated with 50 nM of R16.4 RNA substrate; on the bottom, quantification of nsp15 wt and mutants activity in the presence of R16.4 RNA (% of RNA substrate degraded). The activity of nsp15 wt was considered as 100%. (**C**) 800 nM of nsp15 wt or each mutant version were incubated with 50 nM of 16comp RNA substrate. Reactions were analyzed on 7 M urea/20% polyacrylamide gels. C, control reactions; time points are indicated in the top of each panel. All the experiments were performed at least in triplicate.

FOI 26-3132 - Document 5

nsp15 His235, His250 and Lys290 residues constitute the catalytic triad. Mutating these residues into alanines rendered different results, indicating that they play distinct roles in catalysis. The H235A mutant is almost inactive, while H250A and K290A still retain unspecific activity, similarly to what was observed for the N164A mutant (Figure 5B,C). These results contrast with the ones obtained with MERS-CoV and SARS-CoV nsp15, where changing these residues into alanine rendered a protein with no detectable activity [16,20]. It was already postulated that S294, T341 and Y343 are important for the uridine specificity demonstrated by nsp15 [16,33]. In fact, S294A and Y343A mutants present an unspecific pattern of degradation for both RNA substrates (Figure 5B,C). By quantifying the disappearance of the substrate, we verified that S294A retains ~70% of the activity when compared to the wild-type, whereas the Y343A mutant has twice more activity (Figure 5B). These results confirm that residues S294 and Y343 are, in fact, important for uridine specificity but not for the ability of nsp15 to cleave RNA. Although T341 was also postulated to be involved in this preference of cleavage, our results show that the nsp15 T341A mutant version maintains the specificity for U residues (Figure 5B). However, this substitution affects the ability of nsp15 to efficiently cleave RNA substrates, rendering a protein with half of the activity when compared to the wild-type (Figure 5B). The double mutants (S294A_T341A, T341A_Y343 and S294A_Y343A) exhibited reduced activity and a similar degradation pattern to that observed in the corresponding single mutants, while the triple mutant (S294_T341A_Y343) presented residual activity (Figure 5B,C). These results confirm the importance of these amino acids for nsp15 activity. Altogether, our biochemical analysis points to the importance of several conserved amino acids for nsp15 activity, sequence specificity and oligomerization.

This information is highly valuable to discover compounds that may affect NendoU activity that can be used for the treatment of viral infections provoked by a coronavirus.

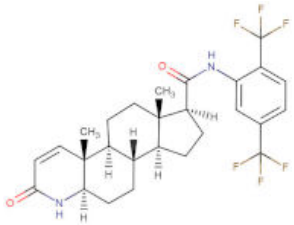
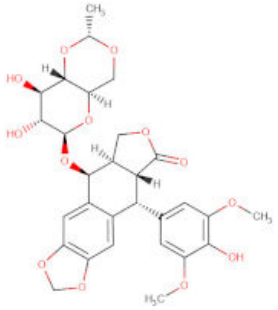
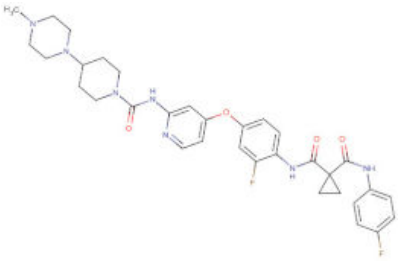
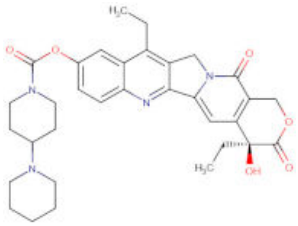
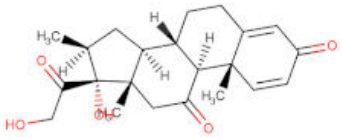
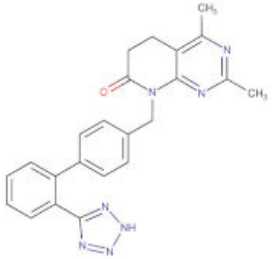
3.4. Searching for SARS-CoV-2 nsp15 Inhibitors

Considering the important role that nsp15 plays in coronavirus pathogenesis, we performed a search for predicted drug candidates that could block the activity of this crucial viral enzyme. We conducted a virtual screening to find inhibitors of nsp15 among 8346 drugs from the Drugbank database that are either approved for clinical use or that are under investigation [27]. Given that the nsp15 activity is increased when incubated in a phosphate-containing buffer, and based on the findings of Kim et al. [31] where nsp15 was co-crystalized with a repurposed drug together with a phosphate ion in the active site, we decided to perform the docking calculations in the presence of a phosphate ion. The drugs with the lowest binding energies from our screening were compared with the ones found by Xu et al. [34]. Six drugs were chosen for further analysis, namely Dutasteride, Irinotecan, Meprednisone, Etoposide, Golvatinib and Tasosartan. The binding affinities ranged from −7.5 kcal/mol up to −9.2 kcal/mol (Table 1). Two binding patterns were observed among the selected drugs. Dutasteride, Golvatinib, Irinotecan and Tasosartan were flexible and long enough to cover the phosphate ion inside the active site (Figure 6A,C,D,F). The extremities of these molecules were located in two regions: one near H235 and another near Y343 and S294. Dutasteride and Golvatinib have fluorinated groups that interact with S294. Together with these halogenated groups, there are aromatic rings that may form pi-stacking interactions with Y343 (Figure 6A,C). Both S294 and Y343 were shown in this study to be important for uridine specificity. Golvatinib is further stabilized by a pi-stacking interaction between another fluorinated aromatic ring with W333. Taken together, these intermolecular interactions may account for the high binding affinity predicted for Golvatinib. Tasosartan also has an aromatic ring near Y343, but it is not well-aligned to form pi-stacking. No specific strong interactions were formed in the case of Irinotecan. Contrary to the first group of molecules, Etoposide and Meprednisone were located only at the top of the active site, interacting with H235 and partially interacting with the phosphate ion (Figure 6B,E). These two molecules did not have specific interactions with nsp15 and were largely exposed to the solvent, which may explain their low binding affinities. The binding modes observed

FOI 26-3132 - Document 5

for the selected drugs are compatible with a scenario of competitive inhibition of nsp15 by occupying the space where the RNA substrate would be bound around the active site.

Table 1. Compounds predicted to bind to nsp15 ribonuclease.

Compound	Category	Binding ΔG (kcal/mol)	Chemical Formula (https://go.drugbank.com, Accessed on 5 of November 2021)
Dutasteride	Approved	−7.8	
Etoposide	Approved	−7.50	
Golvatinib	Investigated	−9.2	
Irinotecan	Approved	−9.0	
Meprednisone	Approved	−7.6	
Tasosartan	Experimental	−8.3	

FOI 26-3132 - Document 5

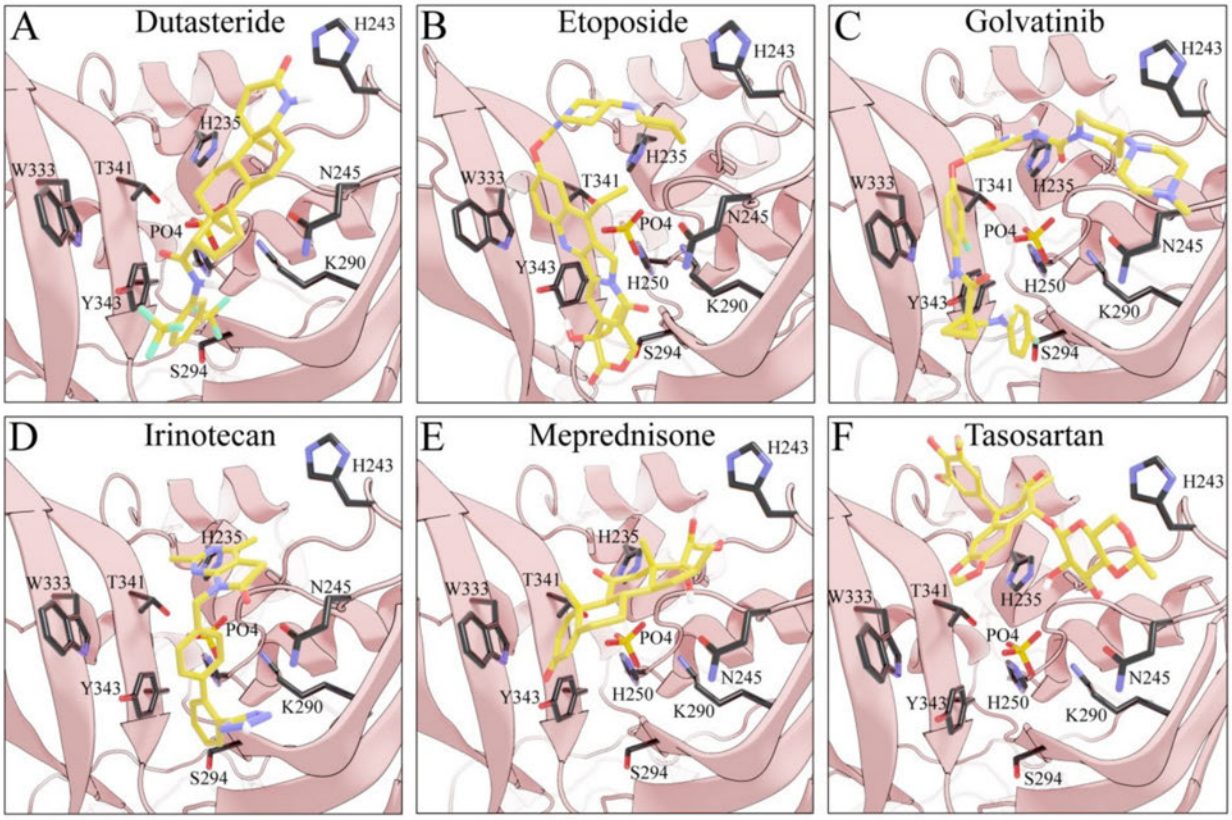


Figure 6. Docking solutions for the six selected drugs (**A**) Dutasteride, (**B**) Etoposide, (**C**) Golvatinib, (**D**) Irinotecan, (**E**) Meprednisone and (**F**) Tasosartan are shown with the carbon atoms in yellow. Key residues of the nsp15 active site are depicted with the carbon atoms in black. The active site and the phosphate ion (PO_4) are covered by Dutasteride, Irinotecan, Golvatinib and Tasosartan. Meprednisone and Etoposide bind only to the top portion of the nsp15 active site.

The selected compounds were tested regarding their ability to inhibit nsp15 activity. In vitro RNA cleavage assays were performed using the R16.4 RNA substrate in the presence or absence of the selected compounds. The RNA and nsp15 concentrations were kept constant. Reaction controls were performed in the absence of the enzymes, with a buffer supplemented with DMSO, since this solvent was used to solubilize all the chemical compounds. To evaluate the biochemical inhibitory effect of the drugs over SARS-CoV-2 nsp15, 1000 µM of each compound were tested. Etoposide, Golvatinib and Irinotecan seemed not to significantly influence the activity of the enzyme in the conditions tested (Figure 7A). However, Dutasteride, Meprednisone and Tasosartan showed ability to inhibit the endoribonuclease activity of nsp15 (Figure 7A). Considering the inibitory effect of these last drugs, we quantified the activity of nsp15 protein in the presence of 600 and 1000 µM of each of the compounds (Figure 7B). In the presence of 600 µM of Dutasteride or Tasosartan, nsp15 retained only ~40% of its activity, while the same concentration of Meprednisone had no effect on the nsp15 activity (Figure 7B). Increasing the concentration of Dutasteride to 1000 µM caused a drastic effect on nsp15 activity, since it almost abolished the ability of the enzyme to cleave the RNA substrate (Figure 7B). 1000 µM of Meprednisone considerably reduced nsp15 activity (Figure 7B). For Tasosartan, the increase in drug concentration had no effect, since the activity was similar to the one detected in the presence of 600 µM (Figure 7B). These results confirmed that nsp15 activity is blocked or significantly reduced in the presence of Dutasteride, Meprednisone and Tasosartan, being Dutasteride the most effective one. These experiments provided important insight into chemical compounds that may be further developed and tested for COVID-19 treatment.

FOI 26-3132 - Document 5

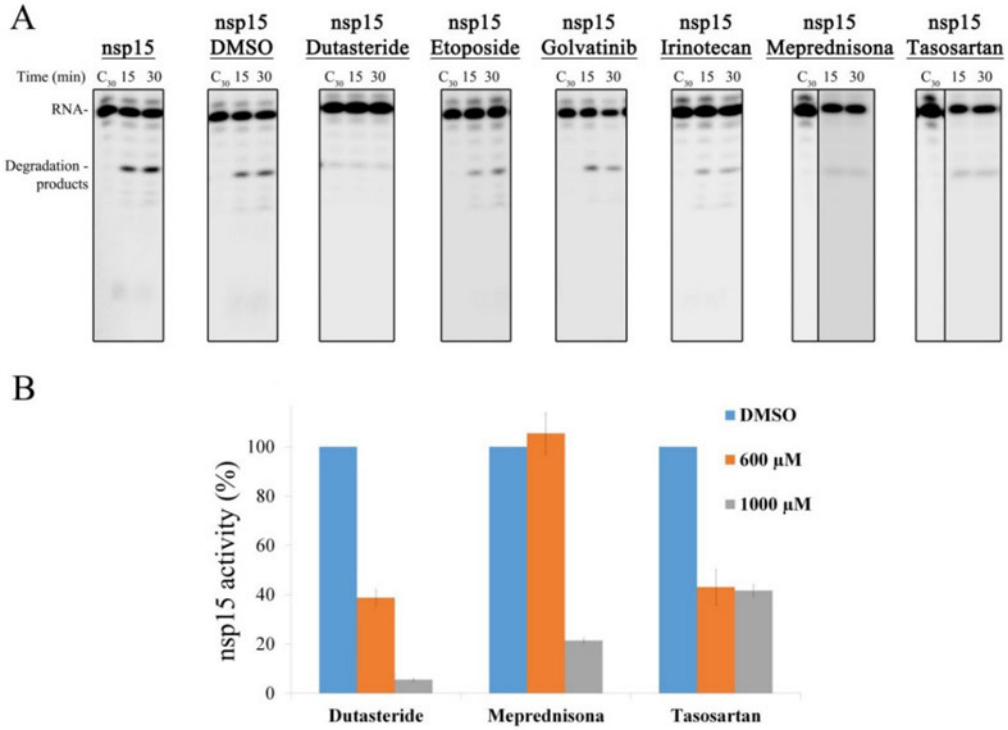


Figure 7. Effect of different drugs in SARS-CoV-2 nsp15 activity. (**A**) 500 nM of nsp15 were incubated with 50 nM of R16.4 RNA substrate in the presence of 1 mM of each drug indicated on the top of the respective panel. Reactions were analyzed on 7-M urea/20% polyacrylamide gels. C, control reactions; time points are indicated in the top of each panel. All the experiments were performed at least in triplicate. (**B**) Quantification of nsp15 activity in the presence of different concentrations of Dutasteride, Meprednisone and Tasosartan. The activity of nsp15 in the presence of DMSO was considered 100%.

4. Discussion

nsp15 is an uridylate-specific endoribonuclease that is highly conserved and present in all coronaviruses [8,11,13,18,35]. This RNase plays a critical role in CoV replication and host immune system evasion, which makes it a very attractive target for drug design [22,36].

In this work, we performed the biochemical characterization of SARS-CoV-2 nsp15 and confirmed its endoribonucleolytic activity [8,13,18,19,30,31,37]. Additionally, we showed different contributions of divalent metal ions for SARS-CoV-2 nsp15 endoribonucleolytic activity. The enzyme showed a preference for Mn^{2+} over Mg^{2+}, Ca^{2+} and Cu^{2+}, while Ni^{2+} and Co^{2+} supported residual activity, and Zn^{2+} inhibited the catalysis. We previously reported that the RNase activity of SARS-CoV-2 nsp14 is also affected by the presence of different metal cofactors [7]. Additionally, Viswanathan et al. described the dependence and preference of SARS-CoV-2 2′-*O*-methyltransferase nsp16 for distinct divalent metal ions [38]. The three enzymes nsp14, nsp15 and nsp16 have a crucial role in innate immune system evasion [22,39,40]. It is tempting to speculate that the surrounding environment could influence the viral response to the host immune system through the modulation of protein activity. Interestingly, Zn^{2+} was shown to inhibit SARS-CoV replication in vitro [41], which is in agreement with our results that demonstrated the inhibition of SARS-CoV-2 nsp15 activity by Zn^{2+}. Moreover, different concentrations of metal ions in blood of patients have been associated with severe illness and the fatal outcome of COVID-19 [42]. This evidence opens the door to explore the biological role of metals in the course of SARS-CoV-2 infection.

nsp15 activity and cleavage specificity have been shown to play important roles in coronavirus life cycles. Our results demonstrated the ability of SARS-CoV-2 nsp15 to cleave single- and double-stranded RNA substrates, with a marked preference for U residues. During CoV replication, the generation of dsRNA intermediates and the

FOI 26-3132 - Document 5

5′-polyuridine (PUN) RNAs produced at the 5′ end of the negative strand are known to trigger type I interferon (IFN-I) [24,43,44]. The endoribonucleolytic activity of nsp15 was found to destroy these intermediate RNAs, hindering the activation of the host innate immune response [22,23,25]. Indeed, nsp15 has been reported as one of the major interferon antagonists from SARS-CoV-2. Thus, it plays a very important role in viral RNA replication and pathogenesis [22,23,25]. Indeed, Nguyen et al. [45] observed that the deletion or inactivation of nsp15 significantly reduced SARS-CoV-2 genome replication. Additionally, in the human Coronavirus HCoV-229E, a nsp15 mutant was found to abolish viral RNA synthesis [8]. In murine hepatitis virus (MHV), nsp15 mutant viruses were defective for viral infection, and deleting parts of the equine arteritis virus (EAV) counterpart rendered a nonviable virus [46,47].

It is known that the biological unit of coronavirus nsp15 homologs is a hexamer, which is crucial for enzymatic activity and RNA binding [16,18–20]. Here, we show that oligomerization is critical for SARS-CoV-2 nsp15 enzymatic activity, being the hexamer of the active form. We also found that the residue N164 is important for nsp15 oligomerization but still retains the ability to cleave RNA; moreover, the oligomeric state seems to impact the U discrimination in SARS-CoV-2 nsp15. In fact, the nsp15 hexamer might be necessary for the stability and organization of the catalytic site and engagement of the RNA molecule. This is the first time that nsp15 oligomerization has been related with its cleavage specificity. Indeed, impacting the efficiency of the enzyme to cleave uridines could be an effective approach to affect viral pathogenesis, since it might have serious consequences in terms of host innate evasion. We also demonstrated that substitution of the residues from the catalytic triad have different outcomes, suggesting a more important role of H235 in catalysis, while the H250A and K290A mutants still retain unspecific activity. This was not observed in the SARS-CoV and MERS counterparts, where these mutations abrogated the activity of nsp15 [16,20]. Thus, our work revealed differences on the functionality of the enzyme that might be related to SARS-CoV-2 pathogenesis. We also demonstrated that the S294 and Y343 residues, which were postulated as responsible for uridine discrimination [16], are, in fact, important for the ability of nsp15 to recognize and cleave U residues but are not critical for the ability of the protein to cleave RNA, as previously described [16,24,33]. Our results indicated that attacking critical nsp15 residues, alone or in combination, could result in an inactive enzyme and, as consequence, a decreased SARS-CoV-2 infectivity. Supporting this, a SARS-CoV-2 replicon system (developed to avoid working with infectious SARS-CoV-2 virus) with a nsp15 H235A_H250A double mutation was shown to have its replication ability highly impaired [45].

Viral infections are known to dramatically alter the host cell metabolism for its replication and spread. SARS-CoV-2 infection was reported to change the metabolic profile in cell lines and lung air–liquid interface cultures [48]. Thus, understanding the influence of metabolites in the course of a viral infection would be extremely valuable. Citrate is a metabolite that plays a key role in pro- and anti-inflammatory homeostasis, contributing to both the antiviral immune response and virus-induced inflammation [49]. In this work, we found that citrate enhances the activity of SARS-CoV-2 nsp15. This finding opens a link between the host cell metabolism and viral RNA degradation pathways. Interestingly, this metabolite was found to co-crystalize with SARS-CoV-2 nsp15 [18]. Citrate binds to the protein active site, promoting its stabilization through Van der Waals contacts with Y343 and hydrogen bonds with the residues H235, H250, K290 and T341 [18], explaining the increase of the endoribonucleolytic activity here observed. It is not the first time that citrate has been reported to modulate the activity of an RNase. The activity of the phosphorolytic ribonuclease PNPase has previously been shown to be inhibited by citrate in the bacteria *E. coli* [50] and *Campylobacter jejuni* (Matos et al., unpublished results). Recently, a structure of SARS-CoV-2 nsp15 bound to Tipiracil was solved, and a phosphate ion was detected in the catalytic pocket [31], which led us to investigate the influence of phosphate over nsp15 activity. Our results demonstrated that phosphate also increases the NendoU activity of SARS-CoV-2 nsp15. It was already described that more than half of the known proteins

FOI 26-3132 - Document 5

interact with phosphate [51]. Additionally, the metabolism of phosphate was suggested to play a critical role in SARS-CoV-2 pathogenesis. For instance, SARS-CoV-2 can induce the depletion of cellular ATP, which consequently interferes with IFN-1 production and induces a cytokine storm [52]. We also discovered that sulfate is capable of inhibiting the endonucleolytic activity of SARS-CoV-2 nsp15. Sulfate ions mimic the phosphate group of RNA and have been used to map enzyme-binding sites [53]. The activity of RNase A, whose active site is structurally similar to that of nsp15, was also found to be inhibited by sulfate [32]. This finding is a significant breakthrough that can be used to rationally design drugs to inhibit nsp15 activity.

Despite the extensive research, the new possible therapies and the already existing vaccines, the worldwide response to the COVID-19 pandemics is still insufficient, and we are lagging behind the virus. In this manuscript, we performed a virtual screening of drugs that could bind the nsp15 active site and block its activity. From the results obtained, we selected the best six drugs to perform in vitro activity assays. Our results demonstrated that Meprednisone, Tasosartan and Dutasteride significantly reduced nsp15 activity, Dutasteride being the most effective compound. Many empirical therapeutic options have been used to treat COVID-19 patients based on the disease progression. Patients with severe COVID-19 can develop a systemic inflammatory response that can lead to lung injury and multisystem organ dysfunction. There are indications that corticosteroids (such as dexamethasone) and other immunosuppressive agents might prevent or mitigate these deleterious effects [54]. Meprednisone is a glucocorticoid and a methylated derivative of prednisone. Prednisone is converted by liver enzymes to the active form prednisolone. Prednisolone is already used to treat a wide range of health problems, such as allergies, blood disorders, skin diseases, infections, cancer, etc. A smaller randomized controlled trial evaluated the effect of methylprednisolone in patients with COVID-19. Although the trial had a low sample size, it showed that the methylated form of prednisolone reduces mortality in hospitalized patients older than 60 years with COVID-19 [55]. Our observation that meprednisone (the methylated and nonmetabolized form of prednisone) has the ability to inhibit the activity of SARS-CoV-2 nsp15 is a good indicator that this drug may exert an antiviral effect. Additionally, its anti-inflammatory properties may also be advantageous to suppress cytokine dysregulation. Tasosartan is used to treat patients with hypertension by blocking the angiotensin II (AT1) receptor and causing vasodilation. It was already reported that tasosartan could be a potential candidate to block the interaction of viral RBD from the spike protein to the ACE2 receptor [56], thus preventing the entry of the virus into the cells. Our work shows that this compound may also prevent viral endoribonucleolytic activity, which is crucial for SARS-CoV-2 replication, being a good candidate to treat COVID-19. Dutasteride is another medication that is primarily used to treat the symptoms of an enlarged prostate. It blocks the conversion of testosterone into dihydrotestosterone, which binds to androgen receptors with a much higher affinity, leading to a poor induction of androgen receptor signaling and to a decrease in human transmembrane protease serine 2 (TMPRSS2) expression. TMPRSS2 was also shown to be necessary for the proteolytic priming of the viral S protein to allow the fusion of SARS-CoV-2 to the cellular membranes. As such, the blocking of the conversion of testosterone into dihydrotestosterone could decrease SARS-CoV-2 entry into human cells [57]. In fact, a double-blinded, placebo-controlled randomized clinical trial (EAT-DUTA AndroCoV) showed that Dutasteride has a beneficial effect in the treatment of COVID-19. Indeed, our results show that this compound has the potential to inhibit the activity of SARS-CoV-2 nsp15. This property might contribute to the reduced viral shedding observed in the trial [58]. However, we should note that Dutasteride is indicated for men and should not be used by women during pregnancy, as it can cause birth defects [59].

One of the great challenges of de novo drug design is to find compounds with desirable pharmacological properties. Therefore, the design of molecules based on scaffolds as the core structures is a way of overcoming this challenge and obtaining potential good drug candidates. Taking this into account, we consider that Meprednisone, Tasosartan and

FOI 26-3132 - Document 5

Dutasteride are excellent scaffolds, due to their effectiveness against nsp15. Two of them are FDA-approved and are being used in patients to treat other diseases, which supports their potential use as drugs. Molecular docking also revealed common features between the selected drugs, namely the interactions with nsp15 S294, Y343 and H235 amino acids, together with their ability to bind to the protein active site in the presence of a phosphate ion. Future designs of efficient drugs could ideally comprise molecules with similar scaffolds to the studied drugs in order to retain most of these features. Additionally, the discovery that sulphate groups are able to inactivate SARS-CoV-2 nsp15 reveals new features to improve or to design new scaffolds.

5. Conclusions

nsp15 has been demonstrating to play an important role in coronavirus pathogenesis. Moreover, there are no closely related homologues to nsp15 in human cells, which makes this ribonuclease a pertinent target for developing CoV inhibitors. nsp15 inhibition may also contribute to revert viral evasion from host cell immunity. Here, we extensively characterized the mechanism of action of SARS-CoV-2 nsp15 activity and added new and useful information for the design/development of therapeutic strategies. Additionally, effective drugs with high therapeutic potential were identified in this work. By using a repurposing approach, in which we tested only drugs that are already approved or under investigation, we may accelerate the use of these compounds as part of a strategy to fight COVID-19 in the very near future. Due to the high homology with other viruses of the family, these results can also be important to fight viruses that may emerge in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10020342/s1, Figure S1: Chromatograms obtained during the size exclusion chromatography step of nsp15 wt and mutants. Table S1: Primers used in this study. Table S2: Plasmids used in this study.

Author Contributions: Conceptualization, M.S. and R.G.M.; methodology, M.S., V.G.C., C.S.S., C.B., S.D., S.C.V., D.L. and R.G.M.; validation, M.S. and R.G.M.; formal analysis, M.S. and R.G.M.; investigation, M.S., V.G.C., C.S.S., C.B., S.D., S.C.V., D.L. and R.G.M.; writing—original draft preparation, M.S. and R.G.M.; writing—review and editing, M.S., V.G.C., C.S.S., C.B., S.D., S.C.V., D.L., C.M.S., C.M.A. and R.G.M.; supervision and funding acquisition, M.S. and R.G.M.,. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by national funds through FCT—Fundação para a Ciência e a Tecnologia, I.P., Project MOSTMICRO-ITQB with refs UIDB/04612/2020 and UIDP/04612/2020, Project PTDC/BIA-BQM/28479/2017 to R.G.M and project PTDC/CCI-BIO/28200/2017 to D.L. and R.G.M and was also financed by a FCT contract (ref. CEECIND/02065/2017). S.C.V. was financed by FCT program IF (ref. IF/00217/2015). M.S., S.D. and D.L. were financed by a FCT contract according to DL57/2016: SFRH/BPD/109464/2015, SFRH/BPD/84080/2012 and SFRH/BPD/92537/2013, respectively. V.G.C. was a recipient of a doctoral grant funded by FCT (ref. 2021.05169.BD). C.B. has a contract under the project PTDC/BIA-BQM/28479/2017. C.S.S. was financed by a fellowship under the project ShikiFactory100, grant agreement number 814408, from the European Union's Horizon 2020 Research and Innovation Program.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We are grateful to the distinguished and experienced virologists Miguel Fevereiro and Margarida Henriques (INIAV, Lisbon, Portugal) for the valuable discussions regarding the physiology of SARS-CoV-2. We also thank Teresa Batista da Silva for the technical support.

Conflicts of Interest: The authors declare no conflict of interest.

FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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COVID-19 outpatients – early risk-stratified treatment with zinc plus low dose hydroxychloroquine and azithromycin: a retrospective case series study

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ABSTRACT

Objective: To describe outcomes of patients with coronavirus disease 2019 (COVID-19) in the outpatient setting after early treatment with zinc, low dose hydroxychloroquine, and azithromycin (the triple therapy) dependent on risk stratification.

Design: Retrospective case series study.

Setting: General practice.

Participants: 141 COVID-19 patients with laboratory confirmed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections in the year 2020.

Main outcome measures: Risk-stratified treatment decision, rate of hospitalization and all-cause death.

Results: Of 335 positively PCR-tested COVID-19 patients, 127 were treated with the triple therapy. 104 of 127 met the defined risk stratification criteria and were included in the analysis. In addition, 37 treated and eligible patients who were confirmed by IgG tests were included in the treatment group (total N=141). 208 of the 335 patients did not meet the risk stratification criteria and were not treated. After 4 days (median, IQR 3-6, available for N=66/141) of onset of symptoms, 141 patients (median age 58 years, IQR 40-67; 73% male) got a prescription for the triple therapy for 5 days. Independent public reference data from 377 confirmed COVID-19 patients of the same community were used as untreated control. 4 of 141 treated patients (2.8%) were hospitalized, which was significantly less ($p<0.001$) compared with 58 of 377 untreated patients (15.4%) (odds ratio 0.16, 95% CI 0.06-0.5). Therefore, the odds of hospitalization of treated patients were 84% less than in the untreated group. One patient (0.7%) died in the treatment group versus 13 patients (3.5%) in the untreated group (odds ratio 0.2, 95% CI 0.03-1.5; $p=0.16$). There were no cardiac side effects.

Conclusions: Risk stratification-based treatment of COVID-19 outpatients as early as possible after symptom onset with the used triple therapy, including the combination of zinc with low dose hydroxychloroquine, was associated with significantly less hospitalizations and 5 times less all-cause deaths.

Keywords: SARS-CoV-2, COVID-19, outpatients, treatment, zinc, hydroxychloroquine, azithromycin

ARTICLE SUMMARY

Strength and limitations of this study

- The first COVID-19 outpatient risk stratification and treatment study
- Repurposed antimalarial drug hydroxychloroquine at low dose in combination with zinc and azithromycin as a therapeutic approach early in the course of COVID-19 until specific drugs or vaccines are available
- Retrospective case series study with findings that have to be validated in prospective controlled clinical trials
- Only outcome data of the untreated control group of the same community based on public reference was available but no other patient characteristics, clinical symptoms, etc.
- No conclusion on the efficacy and safety of the used triple therapy related to severely ill hospitalized patients

INTRODUCTION

In December 2019, the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) started as an outbreak in Wuhan, China. This coronavirus has spread rapidly as a pandemic around the world,[1] causing coronavirus disease 19 (COVID-19) pneumonia, acute respiratory distress syndrome (ARDS), cardiac injury, liver and renal injury, thrombosis, and death.[2]

As of June, 2020, diagnosis and treatment of COVID-19 have been almost exclusively studied from an inpatient perspective, including intensive care with mechanical ventilation. Only one study has described characteristics and key health outcomes of COVID-19 diagnosed patients in an outpatient setting.[3] This is surprising as primary care physicians see COVID-19 patients often first. They could play a critical role in early diagnosis, treatment, and management of disease progression and virus spread. This assumption is supported by the established principle in medicine that speed of eradication is linked to the outcome of life-threatening infections.[4]

The early clinical phase of COVID-19 has not been the focus of research until today even though timing of antiviral treatment seems to be critical.[5] The more optimal window for therapeutic intervention is before the infection spreads from upper to lower respiratory tract and before the severe inflammatory reactions.[6] Therefore, diagnosis and treatment of COVID-19 outpatients as early as possible, even based on clinical diagnosis only, may have been an underestimated first step to slow down or even stop the pandemic more effectively. Based on clinical application principles of antiviral therapies, as demonstrated in the case of influenza A,[7] antiviral treatments should be used early in the course of infection.

Due to the lack of vaccines as well as SARS-CoV-2 specific therapies, the proposed use of repurposed antiviral drugs remains a valid practical consideration.[8] One of the most controversial drugs during the current SARS-CoV-2 pandemic is the well-known oral antimalarial drug hydroxychloroquine (HCQ), routinely used in the treatment of autoimmune diseases like rheumatoid arthritis or lupus.[9] [10] HCQ is currently listed as an essential medication for lupus by the World Health Organization (WHO)[11]. With more than 5.6 million prescriptions in the United States, HCQ was the 128th most commonly prescribed medication in 2017.[12] In the meantime, first observational studies concluding beneficial therapeutic effects of HCQ as monotherapy or in combination with the antibiotic azithromycin were reported just a few weeks after the start of the

SARS-CoV-2 outbreak.[13] All studies that used HCQ with rather contradictory results were done with hospitalized and often sicker patients[13-16] and one publication was recently withdrawn.[17 18] As of June 2020, no studies with COVID-19 outpatients treated with HCQ at an early stage of the disease have been reported.

Antiviral effects of HCQ are well-documented.[19] It is also known that chloroquine and probably HCQ have zinc ionophore characteristics, increasing intracellular zinc concentrations.[20] Zinc itself is able to inhibit coronavirus RNA-dependent RNA polymerase activity (RdRp).[21] It has been hypothesized that zinc may enhance the efficacy of HCQ in treating COVID-19 patients.[22] The first clinical trial results confirming this hypothesis were recently published as preprint.[23] Nevertheless, many studies with HCQ in monotherapy or in combination with the antibiotic azithromycin have been inconclusive so far.[13-16] In all of these studies, HCQ was used later than 5 days after onset of symptoms when hospitalized patients most likely had already progressed to stage II or III of the disease.[6] Regardless of the established antiviral effects of zinc and that many COVID-19 patients are prone to zinc deficiencies, dependent on comorbidities and drug treatments,[22] none of these studies were designed to include zinc supplementation as combination treatment.

This first retrospective case series study with COVID-19 outpatients was done to show whether a) a simple to perform outpatient risk stratification might allow for rapid treatment decision shortly after onset of symptoms, and b) whether the triple 5-day therapy with zinc, low dose HCQ, and azithromycin might result in less hospitalizations and less fatalities compared with relevant public reference data of untreated patients.

METHODS

SETTING

This retrospective case series study analysed data from COVID-19 outpatients with confirmed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in a community in New York State, USA. Outcome of patients who were treated with a specific triple therapy was compared to public reference data of patients in the same community who were not treated with this therapy.

CONFIRMATION OF COVID-19 DIAGNOSIS

COVID-19 diagnosis was confirmed if patients were positively tested for SARS-CoV-2 by means of PCR of nasal or pharyngeal swab specimens (majority of tests by Roche, Basel; 99,1% sensitivity and 99,7% specificity; other tests used with lower frequency included: Diasorin: 500 copies/mL; ThermoFisher: 10 genomic copy equivalents/reaction; Seegene: 1,250 copies/mL; Hologic: TCID50/mL: 1X10-2) or retrospectively by IgG detection tests (DiaSorin: Sensitivity 97.6% (≥ 15 days after diagnosis), specificity 99.3%; Diazyme: Sensitivity 91.2%, specificity 97.3%). Only patients who did have a record of a positive test result were included in the analysis. The PCR assays were authorized by the Food and Drug Administration (FDA) without clinical sensitivity/specificity data due to the urgent nature of the pandemic. Only one positive test was necessary for the patient to be included in the retrospective analysis.

PATIENTS

Sequentially consecutive COVID-19 outpatients older than 18 years at diagnosis were included in the analysis as treatment group. All patients were white. Patients received a prescription for the triple therapy only if they met one of the following risk stratification requirements during a medical office-based or telehealth consultation:

Group A: age >60 years; with or without clinical symptoms;

Group B: age ≤60 years and shortness of breath (SOB);

Group C: age ≤60 years, clinically symptomatic and with at least one of the following comorbidities: hypertension, hyperlipidemia, diabetes, obesity (body mass index ≥ 30 kg/m^2), cardiovascular disease, heart failure, history of stroke, history of deep vein

thrombosis or pulmonary embolism, asthma, chronic obstructive pulmonary disease (COPD), other lung disease, kidney disease, liver disease, autoimmune disease, or history of cancer. Pregnant women, if any, were to be included in this group as well.

Laboratory confirmed COVID-19 patients of the same community who were not treated with the described triple therapy and related outcome data represented the untreated control group (public reference data).

PROCEDURE AND TREATMENT

Data of treated patients was collected from electronic health records in the year 2020. Demographics, as reported by the patient, and a current medical history of hypertension, hyperlipidemia, diabetes, obesity (body mass index ≥ 30 kg/m^2), cardiovascular disease, heart failure, stroke, asthma, COPD, other lung disease, kidney disease, liver disease, autoimmune disease, history of cancer, thyroid disease psychiatric disorder, or pregnancy were collected.

The presence of the following clinical symptoms of treated patients were documented: cough/dry cough, fever, SOB, changes of or no smell or taste, sore throat, headache, runny nose/clear rhinorrea, sinus congestion, diarrhea/vomiting, cold symptoms, feeling sick, weakness, and low back pain. If reported, number of days since onset of symptoms was documented.

The following vital signs, if available, were collected and documented: heart rate (beats per minute), breaths per minute (BPM), systolic and diastolic blood pressure (mmHg), body temperature (°C), oxygen saturation measured by pulse oximetry (O_2 %), body weight (kg), and/or body mass index (BMI).

Main co-medications were characterised based on primary care prescriptions active at the time of diagnosis, were documented as categorical variables and included: beta-blockers, angiotensin-converting enzyme inhibitors, angiotensin-2 antagonists, calcium channel blockers, hydrochlorothiazide, statins, bronchodilators, antidiabetics, and insulin.

Only diagnosed COVID-19 patients who met the defined risk stratification requirements of group A, B, or C got a prescription for the following triple therapy for 5 consecutive days in addition to standard supportive care: zinc sulfate (220 mg capsule once daily, containing 50 mg elemental zinc), HCQ (200 mg twice daily), and azithromycin (500

mg once daily). No loading dose was used. Patients who did not meet the risk stratification requirements received standard of care to treat common upper respiratory infection. Patients were not treated with HCQ if they had known contraindications, including QT prolongation, retinopathy, or glucose-6-phosphate dehydrogenase (G6PD) deficiency. As usual and following best practice patients were informed about possible drug related side effects. Reported events, if any, were documented as required.

The selection of the used zinc supplement and drugs, dosages and the combination thereof, were based on treatment guidelines, positive reports from other countries like South Korea, emerging first clinical evidence, and based on the discretion of the treating physicians.

OUTCOME

Two outcomes were studied: COVID-19 related hospital admission and all-cause death during time of follow up of at least 28 days in the treatment group and in the untreated control group (public reference). The outcome of COVID-19 patients of the untreated control group was reported by the responsible health department.

STATISTICAL ANALYSES

Only patients of the treatment group who met the defined risk stratification requirements and who received at least a prescription for HCQ, with or without zinc, for 5 days, were included in the retrospective analysis and were categorized accordingly. If the patient’s electronic health record did not include information on a clinical characteristic, it was assumed that the characteristic was not present. In the group of the public reference data only confirmed COVID-19 patients who were not treated in the respective general practice with the triple therapy were included in the analysis. For this untreated control group only outcome data for hospitalization and all-cause death was available and used for the statistical comparison with the treatment group.

No sample-size calculations were performed. Descriptive statistics are presented as median and interquartile range (IQR) for continuous variables and frequencies for categorical variables. For comparison with results of other studies means and standard

deviations were calculated as needed. Normality of distribution for continuous variables was assessed by the Shapiro-Wilk-Test. A 2-tailed Student's t-test was used for parametric analysis, and a Wilcoxon Signed-Rank test was used for nonparametric data analysis. For calculation of correlation the point biserial correlation coefficient was applied if one variable was dichotomous. Associations between two categorical variables were calculated with the Chi-Square test. Odds ratio (OR) were calculated for comparison of the outcome of the treatment group with the untreated control group. The α: 0.05 was considered as a significance level. The data were analysed using Microsoft Excel for Microsoft 365 MSO (32-Bit), the Excel add-on Real Statistics, SigmaStat 4, and Sigma Plot 14.0.

PATIENT AND PUBLIC INVOLVEMENT

In this retrospective case series study no patients were involved in the study design or in setting the research questions or the outcome measures directly. No patients were asked to advise on interpretation or writing of results.

STUDY APPROVAL

The study was approved by the Western Institutional Review Board and it was exempt under 45 CFR § 46.104(d)(4). The analysis was conducted with de-identified patient data, according to the USA Health Insurance Portability and Accountability Act (HIPAA), Safe Harbor. For that reason exact dates and locations are not mentioned in this study.

RESULTS

PATIENTS

In accordance with available public reference data, 712 confirmed SARS-CoV-2 PCR positively tested COVID-19 patients were reported for the respective community at the defined time point of the analysis. Of these 712 patients, 335 presented as outpatients at a general practice and 127 were treated with the triple combination therapy. Of these 127 patients, 104 met the risk stratification criteria and were included in the analysis (table 1). 208 patients of the 335 did not meet the defined risk stratification criteria were treated with standard of care and recovered at home. The SARS-CoV-2 infection of 37 additional patients who met the risk stratification criteria and who were also treated with the triple therapy was later confirmed by IgG tests (table 1). These patients were included additionally in the analysis resulting in a total number of 141 patients, all with a confirmed SARS-CoV-2 infection by PCR or IgG tests. None of these patients were lost to follow-up for the defined outcome. The outcome of the remaining N=377 positively tested but not treated COVID-19 patients, e.g. from other practices of the community, served as public reference (fig 1). Analysis of the 141 patients in the treatment group showed that all of these patients (100%) got a prescription of HCQ, 136 (96.5%) of zinc sulfate, and 133 (94.3%) of azithromycin, while 1 patient (0.7%) got doxycycline instead.

BASELINE CHARACTERISTICS OF THE PATIENTS

Table 2 shows the baseline demographics and clinical characteristics of all 141 patients in the treatment group and for the risk stratification groups A, B, and C. 69 patients (49%) belonged to group A, 48 (34%) to group B, and 24 (17%) to group C. Age ranged from 18 to 80 years and the median age was 58 years with an interquartile range (IQR) of 40-67. The median age of group A, B, and C was 67, 39, and 45 years. A total of 103 patients (73.1%) were male with a male-to-female ratio of 2.71. Most common comorbidities included hypertension (28%), obesity (28%), hyperlipidemia (23%), and diabetes (18%), whilst least common ones were liver disease (2%), heart failure (1%), and stroke (1%). One patient was pregnant (1%) at initiation of treatment. There was a positive and significant correlation between age and hypertension ($r=0.3309$, $p=0.001$), hyperlipidemia ($r=0.26306$, $p<0.001$), and cardiovascular disease

(r=0.16757, p<0.05), while asthma was negatively correlated with age (r=-0.30867, p<0.001).

Median time between onset of clinical symptoms and medical consultation was 4 days (IQR 3-6; available for 66/141 patients, mean 4.8 days ± 2.7) (table 3). There was no significant correlation between age and days of onset of clinical symptoms to consultation (p>0.05). Days from onset of symptoms to consultation were not significantly different between groups (p>0.05).

Most common clinical symptoms included cough (87.2%), fever (77.3%), SOB (46.1%), and changes of or no smell or taste (30%), whilst least common ones were sinus congestion (16%), diarrhea/vomiting (5%), and low back pain (3%). Table 4 shows symptoms of all patients and stratified by groups A, B, and C. There was a significant negative correlation between age and changes of smell or taste (r=-0.43, p<0.001). No patient had a clinical diagnosis of pneumonia.

Table 5 shows vital signs, as they were available, for all patients and by group A, B, and C. Many patients consulted the general practice during the COVID-19 crisis via telehealth so vital signs were not available for all of these patients. The highest proportion of patients had available measurements for heart rate (63%) and pulse oximetry (60%). Vital signs were not significantly different between risk stratification groups (p>0.05) except for systolic blood pressure of group A and B (p<0.05).

Table 6 summarizes most important co-medications. 16% of patients were taking angiotensin-converting enzyme inhibitors, angiotensin-2-antagonists, hydrochlorothiazide or a combination thereof. The most common long-term therapies at the time of COVID-19 diagnosis were statins (20%), beta-blockers (12%), and insulin (18%).

HOSPITALIZATIONS AND ALL-CAUSE DEATH

In the treatment group 4 of 141 patients were hospitalized, which was significantly less than in the untreated group with 58 of 377 patients (15.4%), (fig 2.), (OR 0.16; [95% CI, 0.06 to 0.5]; p<0.001), (table 7, fig 4). Therefore, the odds of hospitalization of treated patients were 84% less than in the untreated patients. All hospitalized patients were male, one in his twenties, two in their forties, and one in his seventies. Three of the 4 hospitalized patients (75%) belonged to risk stratification group B and one to

group A (25%). All patients (100%) reported SOB at time of consultation. Median days from onset of symptoms to consultation were 4 days. Of the treatment group 1 patient had to stay only one day in hospital, 2 other patients were discharged as cured, and 1 patient died (s. below). No patient was on a ventilator.

One of the 141 patients (0.71%) who belonged to treatment group A died after being hospitalized. This patient had a history of cancer and did only take one daily dose of the triple therapy before hospital admission. With 13 of 377 patients (3.5%, fig 3) more patients died in the untreated group (OR 0.2; [95% CI, 0.03 to 1.5]) (table 7, fig 4). The odds of all-cause death of treated patients were 80% less (p=0.16) than in the untreated group.

The 208 patients presenting at the general practice who did not meet the risk stratification requirements and who were not treated with the triple therapy recovered at home and no hospital admissions or deaths were reported.

SAFETY

In general, the triple therapy with zinc, low dose HCQ, and azithromycin was well tolerated. After initiation of treatment 30 of 141 patients (21%) reported weakness, 20 (14%) nausea, 15 (11%) diarrhea, and 2 (1%) rash (table 8). No patient reported palpitations or any cardiac side effect.

DISCUSSION

This first retrospective case series study with COVID-19 outpatients in primary care setting showed that risk-stratified treatment early after onset of clinical symptoms, with the triple therapy zinc, low dose HCQ, and azithromycin was associated with significantly less hospitalizations (odds ratio 0.16; p<0.001) and less all-cause deaths (odds ratio 0.2; p=0.16) in comparison to untreated patients (public reference data) of the same community. Based on the performed risk stratification prevalence of the comorbidities hypertension, hyperlipidemia, and diabetes were the highest in group A (>60 years and clinical symptoms), asthma and other lung diseases were the highest in group B (<60 years and SOB), and obesity and autoimmune disease were the highest in group C (<60 years, clinical symptoms, and defined comorbidities). Most frequent symptoms of these COVID-19 patients were cough followed by fever while available median body temperature measurements were in a normal range. Almost 50% of risk-stratified and treated patients were suffering from SOB while breaths per minute and blood oxygen saturation were still in the normal range. Median time from onset of symptoms to first medical consultation was 4 days (IQR 3-6). Approximately 16% of patients received co-medications known to be associated with zinc deficiency, such as antihypertensive drugs. No patient experienced any known severe adverse events that were considered drug related during treatment or follow up.

STRENGTHS AND WEAKNESSES OF THE STUDY

At the time of this manuscript submission, only one peer-reviewed study had analyzed the key health outcomes of COVID-19 patients diagnosed in primary care setting.[3] Because of this gap in data, the value of this study is multifold. It provides much needed recommendations for risk stratification and a treatment regimen to prevent hospitalization and death of COVID-19 patients. Diagnosis of COVID-19 for all patients in this analysis was confirmed by PCR or IgG tests compared with a recent study in which less than 3% had a diagnosis confirmed by laboratory tests.[24] To start the triple therapy as early as possible after symptom onset is critical for treatment success, because SARS-CoV-2 viral load seems to peak at day 5 to 6 after symptom onset[25-27] and severe cases progress to acute respiratory distress syndrome (ARDS) after only 8 to 9 days.[28 29] Early antiviral treatment is an established protocol to manage severe disease progression, as was shown, for example, by a cumulative case control study

during the 2009 H1N1 influenza pandemic in Canada.[30] For patients at high risk for severe viral disease progression, it is recommended to start antiviral therapy as early as possible.[31 32] Early treatment might be also critically important to effectively reduce SARS-CoV-2 viral load,[5] and this underscores the role of early intervention by primary care physicians as reported herein.

Further strength of this approach was the simple risk stratification of symptomatic outpatients to determine the need for therapy, a strategy not yet applied in COVID-19 primary care,[33] but routinely implemented in primary care for other diseases.[34] Underlying assumptions of the risk stratification used in this setting are different than other recommendations.[35] Here, age stratified high risk was defined as >60 years (typically defined as >65 years) to encompass the common increase of comorbidity incidences in this age group.[36] Patients ≤60 years with SOB, even without reduced pulse oximetry values, were treated because it was assumed virus will likely spread from upper to lower respiratory tract.[37] Also treated were patients ≤60 years with clinical symptoms and prognostically relevant comorbidities.[35] By applying this risk stratification approach, respective care was tailored to patients with a higher likelihood for hospitalizations or fatalities, which ensured that the medical principles of “patient first” and “doing no harm” were maintained.[38] As a result, 62% of COVID-19 patients were treated with standard of care only and recovered at home, and only 38% needed treatment with the triple therapy.

The antiviral potential of HCQ was broadly described *in vitro* and *in vivo*.[39-41] HCQ has a long terminal elimination half-life of 32 days in plasma and 50 days in blood.[42] Therefore, the treatment approach was conservative, with starting dose being the same as maintenance dose and with a short treatment duration of only 5 days, being even more conservative than other recommendations.[40] HCQ-dependent intracellular increases in pH might directly interfere with pH-dependent SARS-CoV-2 replication.[19] Also, chloroquine and probably HCQ have characteristics of a zinc ionophore resulting in increasing intracellular zinc concentrations.[20] The dose of elementary zinc in this study was similar to doses previously studied to successfully prevent infections in the elderly.[43] Antiviral effects of zinc against a variety of viruses have been demonstrated during the last decades.[44] Zinc, in addition to its role as a general stimulant of antiviral immunity, is known to specifically inhibit coronavirus RNA-dependent RNA polymerase.[21] Based on HCQ’s ionophore properties, it has been hypothesized that zinc may enhance the efficacy of HCQ in treating COVID-19 patients.[22] In addition,

zinc might inhibit the serine protease furin.[45] Furin is expressed on endothelial cells, monocytes/macrophages, and smooth muscle cells in human atherosclerotic plaques[46] and therefore might play a critical role for the severe cardiovascular complications of COVID-19. As furin might be responsible to favor SARS-CoV-2 spreading compared with other beta coronaviruses[47 48] and as furin-inhibition protects from certain viral-dependent infections[49], it may be important to evaluate the potential role of zinc in inhibiting this pathway.

Azithromycin was added to the treatment regimen as preliminary data provides evidence for more efficient or synergic virus elimination in conjunction with bacterial superinfection.[13 50] Although there is a synergistic antiviral effect between zinc, HCQ, and azithromycin, zinc supplementation may be instrumental for the outcome of patient populations with severe clinical courses. Zinc deficiency was confirmed in a large number of healthy elderly[51] and in diabetic patients.[52] In addition, it has been documented that the antihypertensive drugs hydrochlorothiazide, angiotensin-converting-enzyme inhibitors, and angiotensin 2 receptor antagonists can result in an increased urinary excretion of zinc with subsequent systemic zinc deficiency.[53] Age, comorbidities, and relevant co-medications align well with the majority of described COVID-19 patients at high risk, including the risk-stratified population of this analysis. Zinc deficiency might explain why certain patient groups seem not to benefit from HCQ in monotherapy. During the 5-day treatment with the triple therapy and during follow up, no severe adverse events were observed and no cases of cardiac arrhythmia were reported in this general practice, which is in accordance with available safety data of more than 300,000 patients.[54]

Inherent to all retrospective analyses, our study has certain limitations such as non-randomization and blinding of treatment. Also, only the outcome data of the untreated control group based on the public reference was available but no other patient characteristics or clinical symptoms and so no risk adjustment was possible. Therefore, confounding factors and selection bias, among other issues, do exist. The demographic composition of the treatment group might have also had an influence on our findings. Because many physician appointments had to be managed by telehealth, vital parameters were not available for the majority of patients. Viral load and ECG data were not analyzed. Treatment with the triple therapy resulted in a numerically lower rate of all-cause deaths. In the absence of clinical details about the untreated patient group, the lower rate of all-cause death in the treated group was not statistically

significant. However, the patients in the treated group were all positively risk-stratified while the risk of the untreated group was obviously lower as this group included high- and low-risk patients.

STRENGTHS AND WEAKNESSES IN RELATION TO OTHER STUDIES, DISCUSSING IMPORTANT DIFFERENCES IN RESULTS

In this study, the ratio of males and average age was comparable with a relevant number of other studies, but distribution of comorbidities was not.[55] The latter was expected because outpatients usually have a different distribution of age and especially of comorbidities than critically ill inpatients. As expected the prevalence of hypertension, hyperlipidemia, and cardiovascular disease correlated positively with age while asthma correlated negatively. Approximately 50% of risk-stratified and treated patients presented with SOB while the parameters breaths per minute and blood oxygen saturation were still within the normal range. These patients would usually not be considered for hospital admission, although SOB might be considered an alarming early sign of disease progression. Based on the implemented risk stratification, these patients were identified and treated immediately.

In contrast to many other studies, the most frequent symptom was cough and not fever.[56 57] Changes in smell or taste in one third of patients and a negative correlation with age were similar to findings from other groups.[58] While mean time from onset of symptoms to treatment was only 4.8 days (median 4 days), previously reported time spans range from 6.3 days,[59] to 8 days,[16] up to 16.6 days,[14], or was often even not reported.[60] In most of these studies, COVID-19 disease had most likely already progressed at the time of presentation to stages II or even stage III of the disease.[6] In many studies, often only limited information is provided about co-medications and specifically about clinical symptoms at admission.[60] The latter would be very important to better understand the differences of clinical presentation between inpatients and outpatients, and thus the urgency for early anti-COVID-19 treatment in outpatient setting.[61] The potential of zinc to enhance the antiviral efficacy of HCQ was already described in detail elsewhere.[22] This hypothesis was recently confirmed by a study using a similar triple therapy and treatment duration.[23] Zinc added to HCQ and azithromycin resulted in a significantly increased number of patients being discharged, a reduction in mortality, or transfer to hospice. In another study, when a lower dose of

200 mg HCQ twice daily was added to basic treatment, mortality of even critically ill patients was significantly reduced.[62] These and our findings indicate that proper dosing of HCQ with its long half-life might be key for the favourable outcome of COVID-19 patients. In critical care, drugs with short half-lives are usually preferred. Especially in critically ill COVID-19 patients, higher doses of HCQ may have unforeseeable effects, for example, on insulin sensitivity in obese patients[63] and glucose levels in diabetics.[64] [65] Besides glucose levels, it is important to closely monitor renal function which is increasingly affected during progression of COVID-19.[66] Because HCQ is substantially excreted by the kidneys, the risk of toxic reactions is greater in patients with impaired renal function.[67]

POTENTIAL IMPLICATIONS FOR CLINICIANS AND POLICY MAKERS

Clinical experience from severely ill inpatients with pneumonia who were treated with high dose HCQ are not readily transferable to the outpatient setting with upper respiratory disease only. For outpatients with a median of only 4 days after onset of symptoms, COVID-19 represents a totally different disease and needs to be managed and treated differently.[61] A simple to perform outpatient risk stratification, as shown here, allows rapid treatment decisions and treatment with the triple therapy zinc, low dose HCQ, and azithromycin and may prevent a large number of hospitalizations and probably deaths during the SARS-CoV-2 pandemic. This might also help to avoid overwhelming of the health care systems.

UNANSWERED QUESTIONS AND FUTURE RESEARCH

Almost no general clinical data of COVID-19 outpatients exists and hence responsible experts and stakeholders should ensure a common effort to close this gap by designing studies specifically for primary care setting. Ongoing studies with HCQ should be amended to include combination with zinc. Based on our and others preliminary data, the triple therapy zinc, low dose HCQ, and azithromycin should be used and tested to generate prospective data as soon as possible. As zinc deficiency may play an important role during infection, development, and the clinical course of COVID-19, zinc supplementation in accordance with defined recommended dietary allowances should be evaluated as a simple option for primary prevention. Zinc has a high safety margin

and it would be physiologically already available if for example treatment with HCQ is initiated.

Acknowledgements

We thank all the patients and families involved in this study; the practitioners Dr. Rosy Joseph, Dr. Avery Knapp, Dr. Hillel Isseroff, Dr. William Grace, Dr. Sam Sandowski, and Dr. James Todaro for medical support; Chandra Duggirala, and Manoj Duggirala for operational and technical support; Mendel Mochkin (CrowdProtocol Foundation) for supporting the IRB submission; the reviewers Vjosa C. Mujko (Invivo Brands LLC) and Tzvi Jacobs who improved the language of this publication.

Conflict of interest

The author Roland Derwand is/was at the time of writing an employee of Alexion Pharma Germany GmbH. His engagement and contribution to this study and publication was private and independent from his employer. The author Martin Scholz is/was at the time of writing External Senior Advisor for the company LEUKOCARE in Munich, Germany, and is/was Manging Director at Starts- and -Ups Consulting, Frankfurt, Germany. Vladmir Zelenko is/was general practitioner in New York State. All three authors confirm that this article content has no conflict of interest.

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

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Tables and Figures

Table 1. COVID-19 Diagnostics by PCR and IgG tests of Patients in the Treatment Group				
COVID-19 Diagnostics – no. (%)	**Risk Stratified Group A (N=69)**	**Risk Stratified Group B (N=48)**	**Risk Stratified Group C (N=24)**	**All Patients Treatment Group (N=141)**
SARS-CoV-2 – PCR Test	51 (74)	39 (81)	14 (58)	104 (74)
SARS-CoV-2 – IgG Test	18 (26)	9 (19)	10 (42)	37 (26)

Table 2. Baseline Demographics and Clinical Characteristics of Patients in the Treatment Group*				
Characteristics	**Risk Stratified Group A (N=69)**	**Risk Stratified Group B (N=48)**	**Risk Stratified Group C (N=24)**	**All Patients Treatment Group (N=141)**
Median age (IQR) – years	67 (64-69)	39 (24-47)	45 (36-50)	58 (40-67)
Male sex – no. (%)	46 (67)	40 (83)	17 (71)	103 (73)
Coexisting conditions – no. (%)				
Any condition	44 (64)	31 (65)	24 (100)	99 (70)
Hypertension	27 (39)	4 (8)	8 (33)	39 (28)
Hyperlipidemia	21 (30)	7 (15)	5 (21)	33 (23)
Diabetes	16 (23)	4 (8)	5 (21)	25 (18)
Obesity	20 (29)	10 (21)	10 (42)	40 (28)
Cardiovascular Disease	9 (13)	1 (2)	3 (13)	13 (9)
Heart Failure	2 (3)	0 (0)	0 (0)	2 (1)
Stroke	1 (2)	0 (0)	0 (0)	1 (1)
Asthma	2 (3)	9 (19)	2 (8)	13 (9)
COPD	0 (0)	0 (0)	0 (0)	0 (0)
Other Lung Disease	6 (9)	5 (10)	4 (17)	15 (11)
Kidney Disease	1 (2)	3 (6)	2 (8)	6 (4)
Liver Disease	1 (2)	2 (4)	0 (0)	3 (2)
Autoimmune Disease	2 (3)	4 (8)	4 (17)	10 (7)
History of Cancer	6 (9)	2 (4)	1 (4)	9 (6)
Thyroid Disease	7 (10)	4 (8)	2(8)	13 (9)
Psychiatric Disorder	7 (10)	4 (8)	5 (21)	16 (11)
Pregnancy	-	-	1 (4)	1 (1)

*IQR interquartile range

Table 3. Patients with Reported Days Since Onset of Symptoms in the Treatment Group				
Characteristics	**Risk Stratified Group A (N=69)**	**Risk Stratified Group B (N=48)**	**Risk Stratified Group C (N=24)**	**All Patients Treatment Group (N=141)**
Patients with reported days – no. (%)	32 (46)	25 (48)	9 (38)	66 (47)
Median days since onset of symptoms – (IQR)	4 (3-6)	3 (3-6.5)	4 (3-5.5)	4 (3-6)

Table 4. COVID-19 Diagnostics and Baseline Reported Clinical Symptoms of Patients in the Treatment Group				
Clinical Symptoms – no. (%)	**Risk Stratified Group A (N=69)**	**Risk Stratified Group B (N=48)**	**Risk Stratified Group C (N=24)**	**All Patients Treatment Group (N=141)**
Cough/Dry Cough	60 (87)	39 (81)	24 (100)	123 (87)
Fever	53 (77)	38 (79)	18 (75)	109 (77)
Shortness of Breath (SOB)	17 (25)	48 (100)	0 (0)	65 (46)
Changes of or no smell or taste	21 (30)	19 (40)	2 (8)	42 (30)
Sore Throat	19 (28)	8 (17)	7 (29)	34 (24)
Headache	19 (28)	6 (13)	7 (29)	32 (23)
Runny Nose/Clear Rhinorrhea	16 (23)	8 (17)	4 (17)	28 (20)
Sinus Congestion	10 (15)	9 (19)	4 (17)	23 (16)
Diarrhea/Vomiting	1 (2)	5 (10)	1 (4)	7 (5)
Cold Symptoms	31 (45)	16 (33)	12 (50)	59 (42)
Feels Sick	40 (58)	38 (79)	17 (71)	95 (67)
Weakness	44 (64)	22 (46)	11 (46)	77 (55)
Low Back Pain	3 (4)	0 (0)	1 (4)	4 (3)

Table 5. Physical Examination – Vital Signs of Patients in the Treatment Group		
Parameter		**Patients with available Parameters – no. (%) of N=141**
Median Heart Rate – beats per minute – (IQR)	86 (80-94)	89 (63)
Median Breaths per Minute [BPM] – (IQR)	16 (15-18)	43 (31)
Median Systolic Blood Pressure [mmHg] – (IQR)	126 (120-139)	66 (47)
Median Diastolic Blood Pressure [mmHg] – (IQR)	80 (74-85.5)	66 (47)
Median Body Temperature [°C] – (IQR)	37.2 (37-37.8)	79 (56)
Median Pulse Oximetry [O2 %] – (IQR)	97 (96-98)	85 (60)
Median Body Weight [kg] – (IQR)	88 (72.6-98.4)	43 (31)
Median Body Mass Index [kg/m^2] – (IQR)	32.2 (28.5-36.3)	30 (21)

Table 6. Co-Medications of Patients in the Treatment Group	
Drug Class	**Patients – no. (%) of N=141**
Betablockers	17 (12)
Angiotensin-converting enzyme inhibitors	8 (6)
Angiotensin-2 Antagonists	13 (9)
Calcium channel blockers	8 (6)
Hydrochlorothiazide	6 (4)
Statins	28 (20)
Bronchodilators	10 (7)
Antidiabetics	11 (8)
Insulin	26 (18)

Table 7. Clinical Outcome in the Treated Patient Group versus the Untreated Patient Group					
Outcome	**Treated Group – no. (%) of N=141**	**Untreated Group – no. (%) of N=377**	**Odds Ratio**	**95% CI**	**P-value**
Hospitalization	4 (2.8)	58 (15.4)	0.16	0.06-0.5	<0.001
All-cause death	1 (0.71)	13 (3.5)	0.2	0.03-1.5	0.16

CI=Confidence Interval

Table 8. Summary of Adverse Events	
Event	**Patients – no. (%) of N=141**
Any adverse event	67 (48)
Weakness	30 (21)
Nausea	20 (14)
Diarrhea	15 (11)
Rash	2 (1)

Figure 1

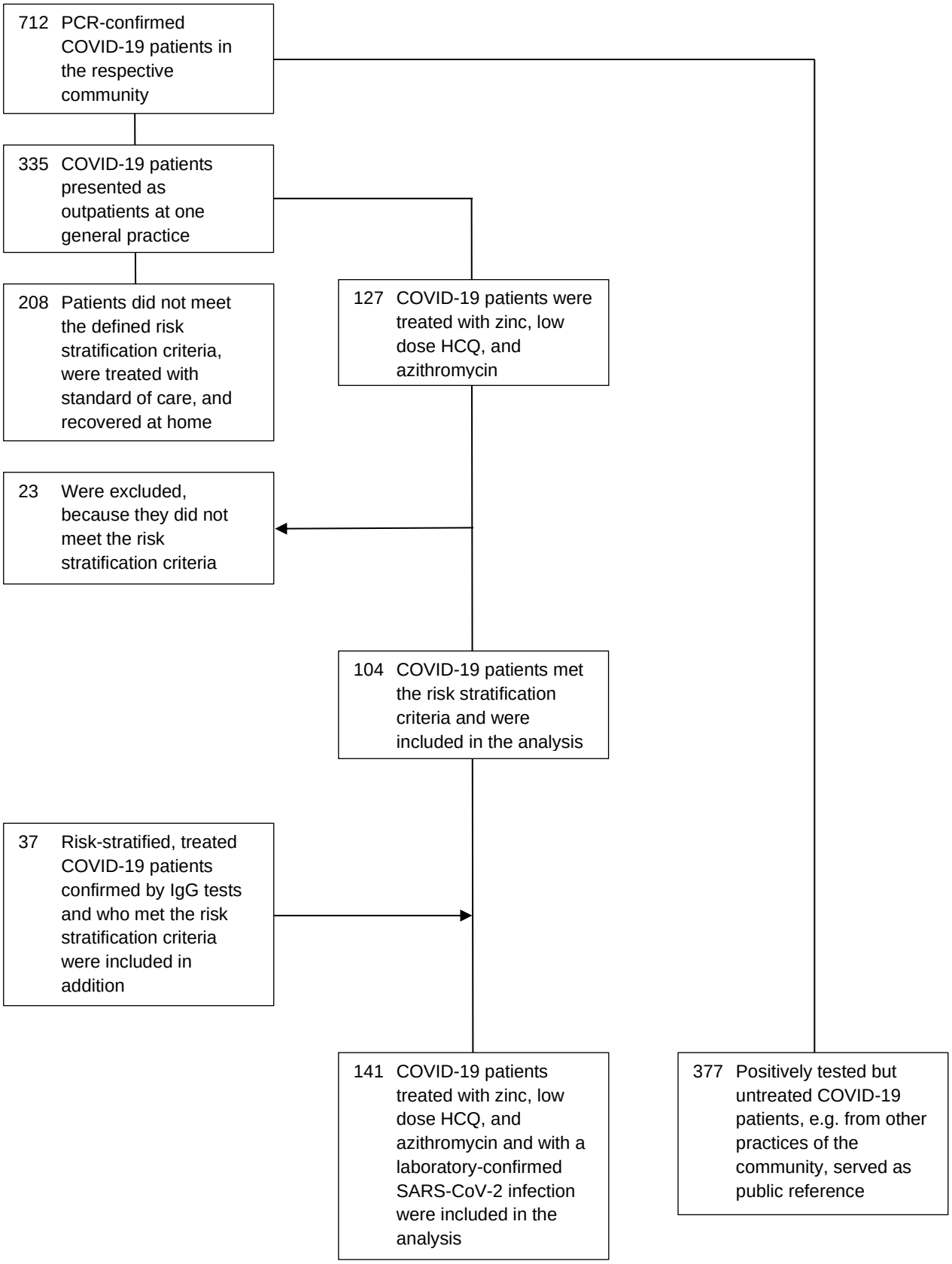


Figure 1: Study population. N=141 COVID-19 patients, all with a laboratory-confirmed SARS-CoV-2 infection, were included in the analysis as treated group. N=377 positively tested COVID-19 patients of the public reference were included in the analysis as untreated group.

Figure 2

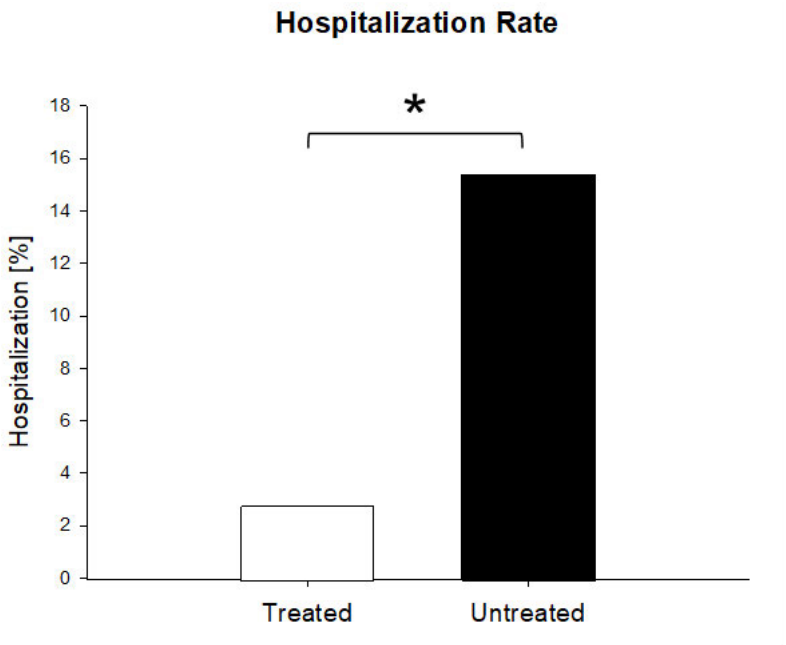


Figure 2: Treatment with the triple therapy zinc, low dose HCQ, and azithromycin was associated with significantly less hospitalizations in comparison to untreated patients of the public reference data. X^2 (1, N=518)=14.17, $^*P<0.001$

Figure 3

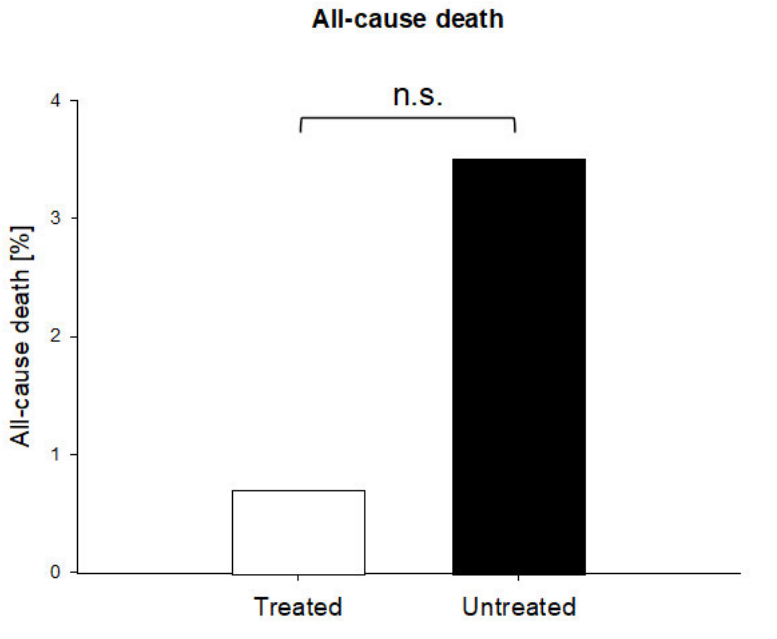


Figure 3: Treatment with the triple therapy zinc, low dose HCQ, and azithromycin was associated with numerically less all-cause deaths in comparison to untreated patients of the public reference data. n.s.=not significant.
X^2 (1, N=518)=1.98, P=0.16

Figure 4

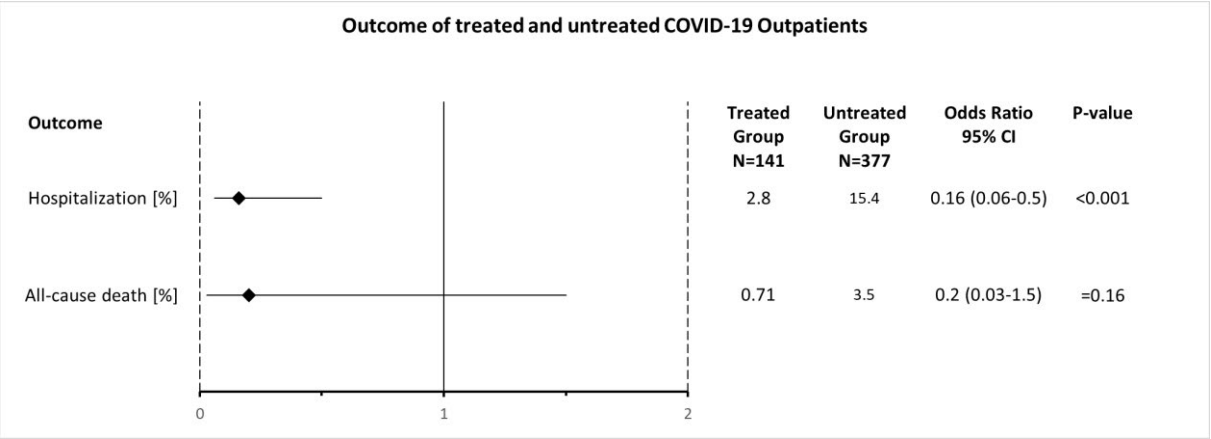


Figure 4: The odds of hospitalization of the treated patient group were 84% less than in the untreated patient group, and was statistically significant (p<0.001). The odds of all-cause death of the treated patient group were 80% less than in the untreated patient group, but did not reach statistical significance (p=0.16). CI=Confidence Interval.

FOI 26-3132 - Document 5

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Cochrane Database Syst Rev. 2015 Apr; 2015(4): CD001364. PMCID: PMC6457799
Published online 2015 Apr 30. doi: 10.1002/14651858.CD001364.pub5 PMID: 25924708

Zinc for the common cold

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Editorial Group: Cochrane Acute Respiratory Infections Group.

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Abstract

Background

The common cold is one of the most widespread illnesses and is a leading cause of visits to the doctor and absence from school and work. Trials conducted in high-income countries since 1984 investigating the role of zinc for the common cold symptoms have had mixed results. Inadequate treatment masking and reduced bioavailability of zinc from some formulations have been cited as influencing results.

Objectives

To assess whether zinc (irrespective of the zinc salt or formulation used) is efficacious in reducing the incidence, severity and duration of common cold symptoms. In addition, we aimed to identify potential sources of heterogeneity in results obtained and to assess their clinical significance.

Search methods

In this updated review, we searched CENTRAL (2012, Issue 12), MEDLINE (1966 to January week 2, 2013), EMBASE (1974 to January 2013), CINAHL (1981 to January 2013), Web of Science (1985 to January 2013), LILACS (1982 to January 2013), WHO ICTRP and clinicaltrials.gov.

Selection criteria

Randomised, double-blind, placebo-controlled trials using zinc for at least five consecutive days to treat, or for at least five months to prevent the common cold.

Data collection and analysis

Two review authors independently extracted data and assessed trial quality.

Main results

FOI 26-3132 - Document 5

Five trials were identified in the updated searches in January 2013 and two of them did not meet our inclusion criteria. We included 16 therapeutic trials (1387 participants) and two preventive trials (394 participants). Intake of zinc was associated with a significant reduction in the duration (days) (mean difference (MD) -1.03, 95% confidence interval (CI) -1.72 to -0.34) (P = 0.003) (I^2 statistic = 89%) but not the severity of common cold symptoms (MD -1.06, 95% CI -2.36 to 0.23) (P = 0.11) (I^2 statistic = 84%). The proportion of participants who were symptomatic after seven days of treatment was significantly smaller (odds ratio (OR) 0.45, 95% CI 0.20 to 1.00) (P = 0.05) than those in the control, (I^2 statistic = 75%). The incidence rate ratio (IRR) of developing a cold (IRR 0.64, 95% CI 0.47 to 0.88) (P = 0.006) (I^2 statistic = 88%), school absence (P = 0.0003) and prescription of antibiotics (P < 0.00001) was lower in the zinc group. Overall adverse events (OR 1.58, 95% CI 1.19 to 2.09) (P = 0.002), bad taste (OR 2.31, 95% CI 1.71 to 3.11) (P < 0.00001) and nausea (OR 2.15, 95% CI 1.44 to 3.23) (P = 0.002) were higher in the zinc group. The very high heterogeneity means that the averaged estimates must be viewed with caution.

Authors' conclusions

Zinc administered within 24 hours of onset of symptoms reduces the duration of common cold symptoms in healthy people but some caution is needed due to the heterogeneity of the data. As the zinc lozenges formulation has been widely studied and there is a significant reduction in the duration of cold at a dose of ≥ 75 mg/day, for those considering using zinc it would be best to use it at this dose throughout the cold. Regarding prophylactic zinc supplementation, currently no firm recommendation can be made because of insufficient data. When using zinc lozenges (not as syrup or tablets) the likely benefit has to be balanced against side effects, notably a bad taste and nausea.

Keywords: Humans, Common Cold, Common Cold/drug therapy, Common Cold/prevention & control, Dosage Forms, Gluconates, Gluconates/adverse effects, Gluconates/therapeutic use, Randomized Controlled Trials as Topic, Severity of Illness Index, Zinc, Zinc/adverse effects, Zinc/therapeutic use, Zinc Acetate, Zinc Acetate/adverse effects, Zinc Acetate/therapeutic use, Zinc Compounds, Zinc Compounds/adverse effects, Zinc Compounds/therapeutic use, Zinc Sulfate, Zinc Sulfate/adverse effects, Zinc Sulfate/therapeutic use

Zinc for the common cold

The common cold is often caused by the rhinovirus. It is one of the most widespread illnesses and is a leading cause of visits to the doctor and absence from school and work. Complications of the common cold include ear infection, sinusitis and exacerbations of reactive airway diseases. There is no proven treatment for the common cold. However, an even partially effective treatment for treating and preventing the common cold could markedly reduce the health problems and economic losses associated with it.

Zinc inhibits replication of the virus and has been tested in trials for treatment of the common cold. This review identified 18 randomised controlled trials, enrolling 1781 participants of all age groups, comparing zinc with placebo (no zinc). We found that zinc (lozenges or syrup) reduces the average duration of the common cold in healthy people, when taken within 24 hours of onset of symptoms. In people taking zinc their cold symptoms are also less likely to persist beyond seven days of treatment. Prophylactic zinc supplementation for at least five months reduces incidence, school absences and prescription of antibiotics for children with the common cold, although antibiotics are not required for the common cold. People in whom common cold symptoms might be troublesome (for example, those with underlying chronic illness, immunodeficiency, asthma, etc.) have not been studied, so the use of zinc cannot currently be recommended for them. Given the variability in the dose, formulation and duration of zinc use in the included studies, more research is needed to address these before zinc use can be generally recommended for the common cold. However, as the zinc lozenges formulation has been widely studied, and there is a significant reduction in the duration of cold at a dose of ≥ 75 mg/day, for those considering using zinc it would be best to use it at this dose throughout the cold. When using zinc lozenges (not as syrup or tablets) the likely benefit has to be balanced against side effects, notably a bad taste and nausea. Regarding prophylactic zinc supplementation, currently no firm recommendation can be made because of insufficient data.

Summary of findings

FOI 26-3132 - Document 5

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Summary of findings for the main comparison

Zinc compared with placebo for the common cold

Patient or population: patients with common cold

Settings: outpatient

Intervention: zinc lozenges or syrup

Comparison: usual care

Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	No. of participants (studies)	Quality of the evidence (GRADE)	Comments
	Assumed risk	Corresponding risk				
	Placebo	Zinc				
Duration of cold symptoms (days)	The mean duration of cold symptoms ranged across control groups from 5.1 to 9.38 days	The mean duration of cold symptoms ranged across control groups from 4 to 12.1 days		1656 (14 studies[1])	++O low[2,3-5]	
Severity of symptom score	The mean severity of symptom score ranged across control groups from 0.4 to 5.61	The mean severity of symptom score ranged across control groups from 0.2 to 3.45		513 (5 studies[6])	++O low[3,5,7,8]	
Incidence of common cold	618 per 1000	382 per 1000 (354 to 431)	RR 0.64 (0.47 to 0.88)	394 (2 studies[9])	+OO very low[3,10-12]	
Number of participants symptomatic after 7 days of treatment	563 per 1000	373 per 1000 (143 to 508)	OR 0.45 (0.2 to 1.0)	476 (5 studies[13])	++OO very low[14-16]	
School absence (number of days)	The mean days of school absence ranged across control	The mean days of school absence in the intervention groups was 0.37		394 (2 studies[9])	+OO very low[10,17,18]	

Open in a separate window

[1]No serious study limitations: all the studies had adequately concealed allocation and blinded both participants and study staff to be considered at low risk of bias. Whether free of other bias was unclear in Macknin 1998; Petrus 1998; Turner 2000a; Turner 2000b; Turner 2000c. Petrus 1998 did not adequately describe the sequence generation. Blinding was inadequate in Turner 2000a; Turner 2000b; Turner 2000c.

[2]Serious inconsistency: there was high statistical heterogeneity. I^2 statistic = 89%. The heterogeneity was due to differences in the nature of the different interventions (zinc gluconate versus acetate lozenges, zinc lozenges versus zinc syrup), wide dose ranges, varied duration of symptoms prior to administration of zinc (varying from 24 to 48 hours) and characteristics of the study population (children versus adults).

[3]No serious indirectness: studies both from low-income and high-income regions have assessed this comparison. Therefore, the result can be confidently generalised to all situations.

FOI 26-3132 - Document 5

[4]No serious imprecision: though the 95% CI around the pooled effect is narrow, the lower limit does not suggest a clinically important reduction in the duration of cold (a decrease in duration of ≤ 1 day is not shown to be important to patients).

[5]Publication bias cannot be ruled out.

[6]No serious study limitation: all the studies had adequately concealed allocation and blinded both participants and study staff to be considered at low risk of bias. Whether free of other bias was unclear and adequate sequence was not generated in one study (Petrus 1998).

[7]Serious imprecision: the 95% CI around the pooled effect is wide, the lower limit is crossing the point of no effect.

[8]Serious inconsistency: there was high statistical heterogeneity. I^2 statistic = 84%. The heterogeneity may be due to differences in the nature of the different interventions (zinc gluconate or acetate lozenges, zinc sulphate syrup) and dose range (30 to 160 mg/day) as well as mean duration of symptoms prior to administration of zinc (varying from 24 to 48 hours), as well as the characteristics of the study population (children versus adults). However, subgroup analysis was not possible as there were not enough studies for each variable.

[9],Kurugol 2006b is a community-based intervention including 200 healthy school children and studying the effect of daily administration of 15 mg zinc sulphate syrup over a period of seven months. Vakili 2009 is also a community-based intervention including 200 healthy school children and studying the effect of daily administration of 10 mg zinc sulfate tablets over a period of seven months.

[10]Serious study limitation: though the study by Kurugol 2006b was of high quality, that by Vakili 2009 was of poor methodological quality.

[11]Serious inconsistency: there is substantial heterogeneity between the two trials: I^2 statistic for heterogeneity = 88%. Both trials showed a benefit with zinc, however the size of this effect was much larger in Vakili 2009. The heterogeneity was due to differences in the trial methodology and the nature of the interventions.

[12]No serious imprecision: the 95% CI around the pooled effect is narrow. Even the lower limit suggests a clinically important reduction in the incidence rate ratio of cold which is shown to be important to patients.

[13]No serious study limitations: allocation concealment was unclear in two studies, i.e. Smith 1989 and Weismann 1990, though both the studies blinded both participants and study staff.

[14]Serious inconsistency: there was high statistical heterogeneity. I^2 statistic = 75%. The heterogeneity may be due to differences in the nature of the different interventions (zinc gluconate or acetate lozenges) and dose range (30 to 160 mg/day) as well as mean duration of symptoms prior to administration of zinc (varying from 24 to 48 hours, as well as the characteristics of the study population (children versus adults). However, subgroup analysis was not possible as there were not enough studies for each variable.

[15]Serious indirectness: only studies from high-income regions have assessed this comparison. Therefore, the result cannot be generalised to all situations.

[16]No serious imprecision: both limits of the 95% CI suggest a clinically important reduction in proportion of participants given the intervention symptomatic after seven days of treatment.

[17]Serious inconsistency: there is substantial heterogeneity between the two trials: I^2 statistic test for heterogeneity = 64%. Both trials showed reduced days of school absence with intervention, however, the size of this effect was much larger in Kurugol 2006b. The heterogeneity was due to differences in the trial methodology and the nature of the interventions.

[18]No serious imprecision: though the 95% CI around the pooled effect is narrow, the lower limit does not suggests a clinically important reduction in the duration of school absence (a decrease in duration of ≤ 1 day is not shown to be important to patients).

[19]No serious inconsistency: there was no statistical heterogeneity. I^2 statistic = 0%.

[20]No serious imprecision: both limits of the 95% CI suggest a clinically important reduction in the rate of antibiotic use with intervention.

[21]No serious study limitations: all the studies had adequately concealed allocation (except Weismann 1990, in which allocation concealment is unclear) and blinded both participants and study staff to be considered at low risk of bias. Whether free of other bias was unclear in Macknin 1998 and Weismann 1990. Weismann 1990 did not adequately describe the sequence generation.

[22]No serious inconsistency: there is no statistical heterogeneity. I^2 statistic = 21%. Both the lozenges and syrup preparation trials were pooled.

[23]No serious imprecision: the 95% CI around the pooled effect is narrow. The resulting adverse events from use of zinc are higher and this is significant.

FOI 26-3132 - Document 5

Background

Description of the condition

The common cold is one of the most widespread illnesses, with adults having two to four episodes annually (Garibaldi 1985). Children may have six to 10 colds a year (and up to 12 colds a year for school children) (Simasek 2007). In the United States, the common cold leads to 75 to 100 million physician visits annually at a conservative cost estimate of USD 7.7 billion per year. Americans spend USD 2.9 billion on over-the-counter drugs and another USD 400 million on prescription medicines for symptomatic relief (Garibaldi 1985; Simasek 2007). More than one-third of patients who saw a doctor received an antibiotic prescription, which has implications for antibiotic resistance from overuse of such drugs (Fendrick 2003). An estimated 22 to 189 million school days are missed annually due to a cold. As a result, parents missed 126 million workdays to stay home to care for their children. When added to the 150 million work days missed by employees suffering from a cold, the total economic impact of cold-related work loss exceeds USD 20 billion per year (Fendrick 2003; Garibaldi 1985). This accounts for 40% of time lost from work (Kirkpatrick 1996). The complications of the common cold include otitis media, sinusitis and exacerbations of reactive airway diseases (Couch 1984; Gwaltney 1966; Turner 2001). Rhinoviruses are the most frequent cause and may account for nearly 80% of common colds during autumn (Turner 2001). There is no proven treatment for the common cold. However, even a medication that is only partially effective in the treatment and prevention of the common cold could markedly reduce morbidity and economic losses due to this illness.

Description of the intervention

The effect of zinc lozenges on the incidence, duration or severity of common cold symptoms has been examined in different studies since 1984. In 1984, Eby et al (Eby 1984) reported for the first time on the efficacy of zinc gluconate lozenges for treatment of the common cold. However, later trials have given variable results. It has been hypothesised that there is a direct correlation between reductions in the duration of common cold symptoms and the daily dosage of all positively charged zinc species released from lozenges at physiologic pH (Eby 1995). The re-analysis of 10 double-blind, placebo-controlled zinc trials by solution chemistry methods showed a significant correlation between total daily dosages of positively charged zinc species and a reduction in the mean duration of common colds (Eby 2004). Zinc gluconate and zinc acetate have very low chemical stability and mainly release positively charged zinc ions in aqueous solutions at physiologic pH, but stronger complexes do not (Eby 2004). Adding a strong zinc binding ligand, such as glycine or citric acid, to a solution containing a zinc complex that is weakly bonded results in the sequestration of zinc to the stronger ligand, reducing or eliminating the benefits of zinc lozenges (Eby 2004). In the review by Marshall it was concluded that zinc gluconate lozenges were effective in reducing the symptoms and duration of the common cold but the side effects and particularly bad taste might limit patient compliance (Marshall 1998). However, results from three trials (Kurugol 2006a; Kurugol 2006b; Kurugol 2007) using zinc sulfate syrup and one trial using zinc sulfate tablet (Vakili 2009) suggested that both the syrup and tablet form are well tolerated and an easy to administer therapy. Adverse effects were mild and had no significant association with the use of zinc sulfate syrup or tablet. The increased incidence of adverse effects noted in the zinc group in various trials may have been related to the use of different ligands (gluconate, acetate) rather than to zinc itself.

How the intervention might work

Interest in the use of zinc for the common cold grew following the results of a randomised controlled trial (RCT) conducted by Eby 1984. Results suggested that if treatment of a cold commenced within three days of the development of cold symptoms and consisted of one 23 mg zinc lozenge dissolved in the mouth every second waking hour, the average duration of cold symptoms was reduced by about seven days. This result was consistent with the earlier observation by Eby (Eby 1984) that a three-year old girl diagnosed with acute lymphocytic leukaemia who had been treated with a 50 mg zinc tablet to improve her zinc status and to stimulate T-cell lymphocyte responsiveness recovered from a cold

FOI 26-3132 - Document 5

within several hours of receiving treatment. In addition, this effect was claimed to be reproducible in other children and adults. Later trials gave inconclusive results (Turner 2001). Results of trials in which no effect of zinc was demonstrated were criticised for having inadequate sample sizes or formulations that reduced the release of zinc ions from the lozenge (Eby 1995).

In-vitro assays indicate that zinc possesses antiviral properties (concentrations of 0.1 mM zinc inhibited growth of eight of nine strains of rhinoviruses) and although such activity suggests Eby's results are biologically plausible, only a handful of RCTs have been able to duplicate his findings. Of the 18 trials conducted since 1984, 11 trials have shown zinc may be useful in the treatment of the common cold and seven have shown no benefit. Most trials showing beneficial effects have been criticised for failing to mask treatment adequately due to the occurrence of side effects, while trials showing no benefit have been criticised for using formulations that reduced the bioavailability of zinc.

Although several possibilities have been suggested, the mechanisms of the efficacy of zinc on the common cold are still unexplained. One possibility is that the interaction of zinc with the host's immune function may have a beneficial effect on common cold symptoms (Macknin 1999). Besides this there is a local mechanism of action: the mouth–nose biologically closed electric circuit (BCEC) which appears to explain the rapid therapeutic response to zinc lozenges (Eby 2010). It moves electrons from the nose into the mouth and, in response to the electron flow, it moves positively charged metal ions, such as ionic zinc, from the mouth into the nose. Human rhinoviruses attaching to the nasal epithelium via the intracellular adhesion molecule-1 (ICAM-1) receptor cause most colds. The zinc ion, based on its electrical charge, has an affinity for ICAM-1 receptor sites and may exert an antiviral effect by attaching to the ICAM-1 receptors in the rhinovirus structure and nasal epithelial cells (Novick 1996). In addition, zinc inhibits viral replication by preventing the formation of viral capsid proteins (Geist 1987; Korant 1976). It has also been suggested that zinc stabilises cell membranes (Pasternak 1987), prevents histamine release (Harisch 1987) and inhibits prostaglandin metabolism (Kelly 1983).

Why it is important to do this review

There is no proven method of prevention or treatment for the common cold. However, any medication that is only partially effective in the treatment and prevention of the common cold could markedly reduce morbidity and economic losses due to this illness. There have been many clinical trials describing the effect of zinc (lozenges and syrup) on common cold symptoms; therefore it is important to know the effect of zinc on the common cold. The last review of all available RCTs of zinc for the common cold was published in 1999. Since then, several new studies (Eby 2006; Kartasurya 2012; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Macknin 1998; McElroy 2003; Petrus 1998; Prasad 2000; Prasad 2008; Turner 2000a; Turner 2000b; Turner 2000c; Vakili 2009; Veverka 2009) have been published. It is therefore important to update the information and include all new clinical trials. We undertook the review to assess the overall effectiveness of zinc (lozenges or syrup) in treating the common cold and to provide some guidance with respect to future research.

Objectives

To assess whether zinc (irrespective of the zinc salt or formulation used) is efficacious in reducing the incidence, severity and duration of common cold symptoms. In addition, we aimed to identify potential sources of heterogeneity in results obtained and to assess their clinical significance.

Methods

Criteria for considering studies for this review

Types of studies Double-blind, placebo-controlled randomised controlled trials (RCTs).

Types of participants Trial participants were of either gender and of any age.

Types of interventions Therapeutic trials: interventions commenced within three days of participants developing common cold symptoms and consisted of 1.5 to 2-hourly treatments with a zinc or placebo lozenge during waking hours, for more than six hours a day for a period of five or more consecutive

days.

Prophylactic trials: intervention commenced and continued throughout the cold season for at least five months.

We considered all formulations of zinc (irrespective of the type of salt, formulation and concentration of zinc).

Types of outcome measures Outcome measures frequently used to determine the clinical efficacy of any common cold treatment are the incidence, severity and duration of cold symptoms. Accordingly, for inclusion in this review, the incidence and severity of at least throat and nasal symptoms and cough needed to be assessed.

Primary outcomes

1. Duration of symptoms.
2. Severity of symptoms.
3. Incidence of the common cold.

Secondary outcomes

1. Proportion of participants symptomatic after three, five or seven days of treatment.
2. Time to resolution of individual symptoms: cough, nasal congestion, nasal drainage and sore throat.
3. Change in individual severity symptom scores: cough, nasal score.
4. School absence (days).
5. Antibiotic use.
6. Adverse events.

We defined duration as the number of days to cold resolution from start of treatment. We considered cold resolution to be the resolution of all cold symptoms or resolution of all but one cold symptom, or the participant believed they had recovered from the cold. Severity of cold symptoms needed to be graded: 0 - no symptoms, 1 - mild symptoms, 2 - moderate symptoms and 3 - severe symptoms. We defined incidence as number of colds per study participant during the study period. Adverse events included any or individual adverse events during or after taking the medications.

Search methods for identification of studies

Electronic searches For this 2013 review update we updated the searches in CENTRAL, MEDLINE and EMBASE and in addition searched CINAHL, Web of Science and LILACS.

We searched the Cochrane Central Register of Controlled Trials (CENTRAL) 2012, Issue 12, part of *The Cochrane Library*, www.thecochranelibrary.com (accessed 18 January 2013), which contains the Acute Respiratory Infections Group's Specialised Register, MEDLINE (April 2010 to January week 2, 2013), EMBASE (1974 to January 2013), CINAHL (1981 to January 2013), Web of Science (1985 to January 2013) and LILACS (1982 to January 2013). See details of search strategy in Appendix 1. Details of the previous search strategy are in Appendix 2.

Searching other resources We searched the US National Institutes of Health, Department of Health and Human Services trials registry www.clinicaltrials.gov and the WHO ICTRP trials registry http://www.who.int/ictrp/en/ (18 June 2012). We also searched bibliographies of published papers for unpublished trials. Two review authors (RRD, MS) assessed the studies to ensure appropriate trials were included in the review and to minimise the potential for selection bias.

Data collection and analysis

FOI 26-3132 - Document 5

More information on the statistical methods used in this review can be found in the relevant section of the Cochrane Acute Respiratory Infections Review Group Module. Comparisons were zinc (lozenges or syrup or tablet) with placebo. We compared outcome measures before and after treatment, as well as after day three, five or seven to accommodate trials of different lengths.

Selection of studies Two review authors (RRD, MS) independently reviewed the results for inclusion in the analysis. We resolved differences regarding study quality through discussion.

Data extraction and management We recorded data on a pre-structured data extraction form. The lead review author (MS) entered data directly into Review Manager (RevMan) (RevMan 2012). An independent coder verified accuracy of data entry. We made no attempt to contact investigators. Most trials were conducted over 10 years ago and in view of the information required to be provided by the investigators, we thought that they would be unable to comply.

Assessment of risk of bias in included studies We assessed risk of bias in all included studies using The Cochrane Collaboration's 'Risk of bias' methodology (Higgins 2011). Two review authors (RRD, MS) assessed selection bias (random sequence generation, allocation concealment), performance bias (blinding of participants and personnel), detection bias (blinding of outcome assessment), attrition bias (incomplete outcome data), reporting bias (selective reporting) and other biases, if any. Each item was assessed as high, low or unclear risk of bias along with relevant information reported in the RCT. When the methodological description was unambiguous, one review author entered the methodological description in the 'Risk of bias' table. When the description of methods was ambiguous, the same review author discussed the issue with the co-author to reach a consensus. The methodological descriptions are summarised in Figure 1 and Figure 2.

FOI 26-3132 - Document 5

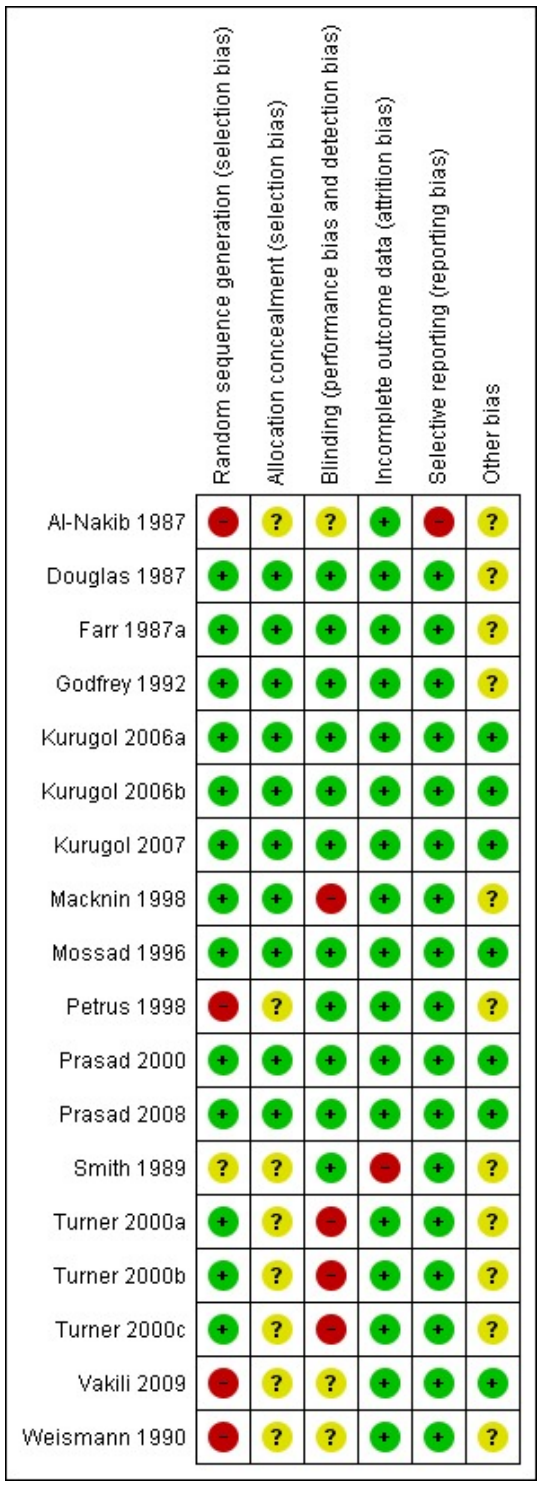


Figure 1

Methodological quality summary: review authors' judgements about each methodological quality item for each included study.

FOI 26-3132 - Document 5

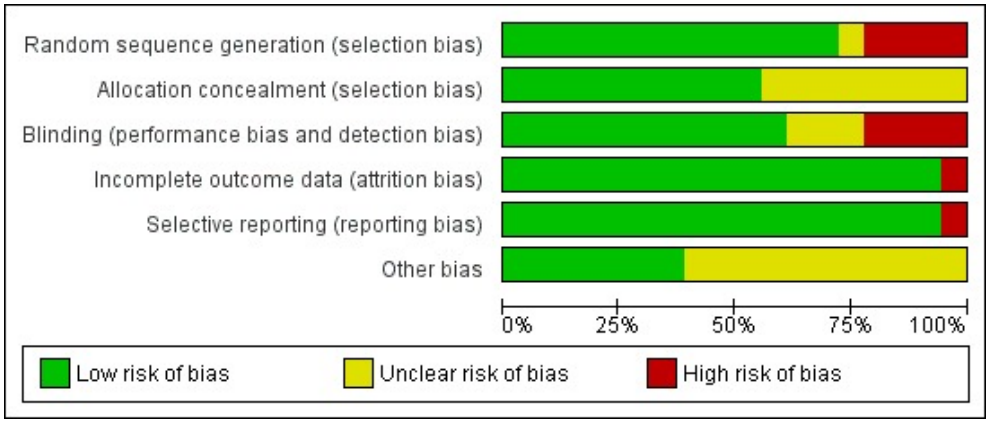


Figure 2

Methodological quality graph: review authors' judgements about each methodological quality item presented as percentages across all included studies.

Measures of treatment effect We extracted outcome data and entered data into RevMan 2012 for statistical analysis. We used the standard methods of the Cochrane Acute Respiratory Infections (ARI) Review Group to synthesise the data. For dichotomous data, we calculated a pooled estimate of the treatment effect for each outcome across trials using the odds ratio (OR). But, for the measurement of incidence of common cold, the incident rate ratio (IRR) was calculated and expressed as RR to match it with the IRR. For continuous outcomes, we recorded both mean post-treatment or post-intervention values and standard deviation (SD). If standard errors (SE) had been reported (and if it were possible) we planned to convert these to standard deviations. We calculated a pooled estimate of treatment effect by calculating either the mean difference (MD) or standardised mean difference (SMD). For both dichotomous and continuous outcomes, we calculated the 95% confidence interval (95% CI) for individual studies. We used fixed-effect models to obtain summary statistics of all types of outcome measures when there was low degree of heterogeneity. When significant heterogeneity was found, we calculated the overall efficacy using random-effects models, which provided more realistic estimates of the CIs under these circumstances (Lau 1997). In this context, a P value < 0.05 indicated significant differences between studies and raised questions as to whether the results could be meaningfully combined. Where it was not possible to perform a meta-analysis, we summarised the data for each trial.

Unit of analysis issues We included only randomised, double-blind, placebo-controlled trials in this review. None of the trials were cross-over or cluster-randomised trials.

Dealing with missing data As many trials were conducted 10 years ago, we thought that the investigators would be unable to compile the missing data, so we did not contact them. For all the outcomes, we considered that incomplete outcome data had been adequately addressed if 80% or more of the participants were included in the analysis, or if less than 80% were included but adequate steps were taken to ensure or demonstrate that this did not bias the results. We performed intention-to-treat (ITT) analysis where the above was not clear. In trials with missing statistics (such as SDs or correlation coefficients), we calculated the data from the available information.

Assessment of heterogeneity We assessed the degree of heterogeneity by using the Chi^2 test and the I^2 statistic (Higgins 2003; Higgins 2011). The Chi^2 test is known to be poor at detecting true heterogeneity among studies; while a statistically significant result indicates heterogeneity, a non-significant result is not evidence of no heterogeneity. The I^2 statistic describes the percentage of total variation across studies that is due to heterogeneity rather than chance. The values of the I^2 statistic lie between 0% and 100%. For the current meta-analysis, we used a simplified categorisation of the I^2 statistic as follows: if significant heterogeneity (I^2 statistic > 50%) was found, we used a random-effects model and if low heterogeneity (I^2 statistic < 50%) was found, we used a fixed-effect model.

FOI 26-3132 - Document 5

Assessment of reporting biases We sought further information from trial authors, although this was not possible for the current meta-analysis as many of the studies were very old. We looked hard for evidence of collection by study investigators of a small number of key outcomes that are routinely measured in the area in question, and reported which studies reported data on these and which did not. We also constructed a matrix indicating which outcomes were recorded in which studies (for example, with rows as studies and columns as outcomes). Complete and incomplete reporting was also indicated. This matrix showed us which studies did not report outcomes reported by most other studies. We assessed risk of bias due to selective reporting of outcomes for the study as a whole as well for each outcome. We also assessed the likelihood of small study effects, such as publication bias, by examining the funnel plot for asymmetry (Egger 1997).

Data synthesis We analysed data using a fixed-effect model in cases of low heterogeneity (I^2 statistic value of < 50%) and a random-effects model in cases of moderate to high heterogeneity between studies (I^2 statistic value of > 50%).

Subgroup analysis and investigation of heterogeneity We considered the following factors as possible explanations for the heterogeneity observed across the results of these studies: dosage and formulations of zinc used, age of participants (children and adults) and the mean duration of symptoms prior to administration of zinc. We plan to investigate these with subgroup analyses when there are sufficient included studies.

Sensitivity analysis We had planned to perform sensitivity analyses based on methodological quality of the trials (with and without quasi-randomised trials), but this was not possible as all were randomised, double-blind, placebo-controlled trials.

Results

Description of studies

Results of the search We retrieved 77 additional studies (MEDLINE = 16, CENTRAL = 11, CINAHL = 41, Web of Science = 77, EMBASE = 15 and LILACS = 4) after removing duplicates in the new searches conducted between June 2010 and January 2013. Two review authors (RRD, MS) screened the new search results. Out of 77 new searches, one trial was screened for potential eligibility after reading the abstract. However, this trial (Kartasurya 2012) did not meet the inclusion criteria and was later excluded.

Included studies We included three additional trials (Turner 2000a; Turner 2000b; Turner 2000c) that were previously excluded, based on unclear methodology. Besides this, no new trials contributed to the data synthesis in this updated review. We included 18 trials (1387 participants in the therapeutic trials and 394 in the preventive trials) in this review.

Location Three trials were conducted in Turkey (Kurugol 2006a; Kurugol 2006b; Kurugol 2007); one trial each in Iran (Vakili 2009), Denmark (Weismann 1990), UK (Al-Nakib 1987) and Australia (Douglas 1987); and 11 trials in the USA (Farr 1987a; Godfrey 1992; Macknin 1998; Mossad 1996; Petrus 1998; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c).

Participants All the trial participants included in the analysis were both adults and children with age range varying from one to 65 years at the start of the trials. Five trials included children only and among these three trials included children aged one to 10 years (Kurugol 2006a; Kurugol 2006b; Kurugol 2007); one included children and adolescents aged six to 16 years (Macknin 1998) and another included children aged 6.5 to 10 years (Vakili 2009). Two trials recruited participants from volunteers experimentally inoculated with human rhinovirus (Al-Nakib 1987; Farr 1987a). Given that not all participants had cold symptoms at the beginning of the intervention in one trial (Farr 1987a), this trial would be excluded from the statistical overview. In one trial neither the health status of the participants nor the exclusion criteria were stated, while in other trials only healthy subjects from the general population were included. In one trial, participants with cold durations of more than 24 hours (Macknin 1998) and in another trial participants with cold durations of more than 48 hours (Kurugol 2007) were excluded. The trials varied widely in size; two trials had fewer than 50 participants (Prasad 2000;

FOI 26-3132 - Document 5

Prasad 2008), four had more than 50 but fewer than 100 participants (Douglas 1987; Farr 1987a; Godfrey 1992; Mossad 1996), eight had more than 100 but fewer than 200 participants (Al-Nakib 1987; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Petrus 1998; Smith 1989; Weismann 1990; Vakili 2009) and four had more than 200 participants (Macknin 1998; Turner 2000a; Turner 2000b; Turner 2000c).

Interventions Zinc supplements were provided in the form of either syrup, lozenges or tablets. One trial used zinc sulfate tablet (Vakili 2009) and three trials used zinc sulphate syrup (Kurugol 2006a; Kurugol 2006b; Kurugol 2007). Among the trials using lozenge preparations, two different salts were used: zinc gluconate (Al-Nakib 1987; Farr 1987a; Godfrey 1992; Macknin 1998; Mossad 1996; Smith 1989; Weismann 1990; Turner 2000a) and zinc acetate (Douglas 1987; Petrus 1998; Prasad 2000; Prasad 2008; Turner 2000b; Turner 2000c). The supplements were given for different periods of time in all the trials. In the therapeutic trials the duration of supplement was five days (Farr 1987a), six days (Al-Nakib 1987; Douglas 1987), seven days (Farr 1987a; Godfrey 1992; Smith 1989), 10 days (Kurugol 2006a; Kurugol 2007; Weismann 1990), 14 days (Petrus 1998) and no duration mentioned (i.e. participants were given zinc as long as they were symptomatic) (Macknin 1998; Mossad 1996; Prasad 2000; Prasad 2008; Turner 2000a; Turner 2000b; Turner 2000c). In the three trials also studying the prophylactic role of zinc, the duration of supplement was 4.5 days (Al-Nakib 1987), five months (Vakili 2009) and seven months (Kurugol 2006b).

Outcomes

Primary Fourteen trials (Douglas 1987; Godfrey 1992; Kurugol 2006a; Kurugol 2007; Macknin 1998; Mossad 1996; Petrus 1998; Prasad 2000; Prasad 2008; Smith 1989; Weismann 1990; Turner 2000a; Turner 2000b; Turner 2000c) reported the duration of symptoms. Among these, five trials have provided the original data (Kurugol 2006a; Kurugol 2006b; Petrus 1998; Prasad 2000; Prasad 2008). The mean and SD were calculated either from the survival curves (Macknin 1998; Mossad 1996; Prasad 2000; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990) or from t/P value (Douglas 1987; Godfrey 1992) reported in other trials. Thirteen trials measured the total severity score of cold symptoms (Al-Nakib 1987; Douglas 1987; Godfrey 1992; Kurugol 2006a; Kurugol 2007; Petrus 1998; Prasad 2000; Prasad 2008; Smith 1989; Weismann 1990; Turner 2000a; Turner 2000b; Turner 2000c) but results from only five trials (Kurugol 2006a; Kurugol 2007; Petrus 1998; Prasad 2000; Prasad 2008) could be pooled, as in eight trials (Al-Nakib 1987; Douglas 1987; Godfrey 1992; Smith 1989; Weismann 1990; Turner 2000a; Turner 2000b; Turner 2000c) the results were not reported in a standard format. The incidence of cold symptoms was measured in two trials (Kurugol 2006b; Vakili 2009).

Secondary The proportion of participants asymptomatic by day three or day five was reported in three trials (Mossad 1996; Smith 1989; Weismann 1990), whereas the proportion of participants asymptomatic by day seven was reported in five trials (Douglas 1987; Godfrey 1992; Mossad 1996; Smith 1989; Weismann 1990). In all these trials, ITT analysis was conducted. Time to resolution of individual cold symptoms was reported as follows: time to resolution of cough in four trials (Kurugol 2006a; Macknin 1998; Prasad 2000; Prasad 2008), time to resolution of nasal congestion in five trials (Kurugol 2006a; Macknin 1998; Petrus 1998; Prasad 2000; Prasad 2008), time to resolution of nasal drainage in five trials (Kurugol 2006a; Macknin 1998; Petrus 1998; Prasad 2000; Prasad 2008) and time to resolution of sore throat in four trials (Kurugol 2006a; Macknin 1998; Prasad 2000; Prasad 2008). Change in individual severity symptom score was reported as follows: change in cough symptom score in two trials (Douglas 1987; Petrus 1998), change in nasal symptom score in four trials (Douglas 1987; Kurugol 2006a; Kurugol 2007; Petrus 1998), change in throat symptom score in two trials (Douglas 1987; Petrus 1998). Standard error of mean (SEM) was not provided in one trial (Douglas 1987). Effect on school absence and antibiotic use were provided in two trials (Kurugol 2006b; Vakili 2009).

Adverse events Fourteen trials (Douglas 1987; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Macknin 1998; Mossad 1996; Prasad 2000; Prasad 2008; Smith 1989; Weismann 1990; Vakili 2009; Turner 2000a; Turner 2000b; Turner 2000c) reported adverse events. Common adverse events included bad taste, nausea, constipation, diarrhoea, abdominal pain, dry mouth and oral irritation. We reported the adverse events separately for both lozenges and syrup formulations.

FOI 26-3132 - Document 5

Other Six trials using experimentally induced colds with rhinovirus also studied the number of participants shedding the virus, duration of viral shedding, number of virus-positive days, as well as rise in antibody titre. These were not included in the outcome measures as we thought that it would not be of help in drawing conclusions. Three trials reported the effect of zinc supplementation on school absence. Among these, two (Kurugol 2006b; Vakili 2009) reported this outcome during a prophylactic trial, though another (Macknin 1998) was a therapeutic trial.

Excluded studies We excluded five trials.

1. Inclusion criteria not defined, disproportionate number of drop-outs from the zinc group (Eby 1984).
2. Two studies were not RCTs (McElroy 2003).
3. Measured upper respiratory tract infection as a whole (including common cold, seasonal influenza) (Veverka 2009).
4. Used both zinc gluconate nasal spray and zinc orotate lozenges simultaneously (Eby 2006).
5. Studies upper respiratory tract infection as a whole, used zinc supplementation for four months (Kartasurya 2012).

Risk of bias in included studies

Allocation Allocation concealment was adequate in 10 studies (Douglas 1987; Farr 1987a; Godfrey 1992; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Macknin 1998; Mossad 1996; Prasad 2000; Prasad 2008). It was unclear in seven studies (Al-Nakib 1987; Petrus 1998; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990) and not described in one (Vakili 2009).

Adequate sequence generation was described in seven studies (Douglas 1987; Godfrey 1992; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Macknin 1998; Mossad 1996). However, it was not clear in seven studies (Farr 1987a; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c) and not generated in four studies (Al-Nakib 1987; Petrus 1998; Vakili 2009; Weismann 1990).

Blinding All 18 studies were blinded but placebo blinding was adequately described in 10 trials (Douglas 1987; Godfrey 1992; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Macknin 1998; Mossad 1996; Prasad 2000; Prasad 2008; Smith 1989). Zinc-treated participants also experienced higher incidences of side effects and/or complaints, and in 12 trials, zinc-treated participants complained of altered, bad or unpalatable taste which suggests that the zinc lozenges were distinct from the placebo lozenges and, in this respect, blinding may have been compromised.

Incomplete outcome data Data were fully detailed in 15 studies and in the remaining three studies (Al-Nakib 1987; Turner 2000a; Turner 2000b; Turner 2000c) details of attrition and exclusions from the analysis were unavailable.

Selective reporting Except two studies (Al-Nakib 1987; Weismann 1990), 16 studies scored 'yes' for being free from selective reporting.

Other potential sources of bias Eleven studies were funded by pharmaceutical companies (Al-Nakib 1987; Douglas 1987; Godfrey 1992; Farr 1987a; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Macknin 1998; Petrus 1998; Smith 1989; Weismann 1990). Five studies were supported Medical Research Foundation (Godfrey 1992; Mossad 1996; Prasad 2000; Prasad 2008; Vakili 2009) and in addition by National Institute of Health (NIH) (Prasad 2008). Information on clearance by Ethics Committees or Institutional Review Boards was available for all except one study (Smith 1989). In three studies, other sources of bias were not clear (Turner 2000a; Turner 2000b; Turner 2000c).

Effects of interventions

See: Table 1

1. Primary outcomes

(i) Therapeutic effects of zinc

FOI 26-3132 - Document 5

Duration of cold symptoms Fourteen studies (Godfrey 1992; Kurugol 2006a; Kurugol 2007; Macknin 1998; Mossad 1996; Petrus 1998; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990) reported this outcome. Results could be pooled from all the studies and there were 1656 participants including both children and adults (Figure 3; Analysis 1.1). The studies were heterogenous in terms of variable formulations (zinc gluconate or acetate lozenges, zinc sulphate syrup) and dose range (30 mg/day to 160 mg/day) as well as MD of symptoms prior to administration of zinc (varying from 24 to 48 hours). Intake of zinc lozenges or syrup was associated with a significant reduction in the duration (days) of common cold (mean difference (MD) -1.03, 95% confidence interval (CI) -1.72 to -0.34) (P = 0.003) when it was administered within 24 hours of the onset of symptoms. However, there was marked heterogeneity among the included trials. We did subgroup analysis for the following: dose (≥ 75 mg/day versus < 75 mg/day), types of lozenges (gluconate versus acetate), formulation (lozenges versus syrup) and age group (children < 16 years versus adults) (Figure 4; Analysis 1.2).

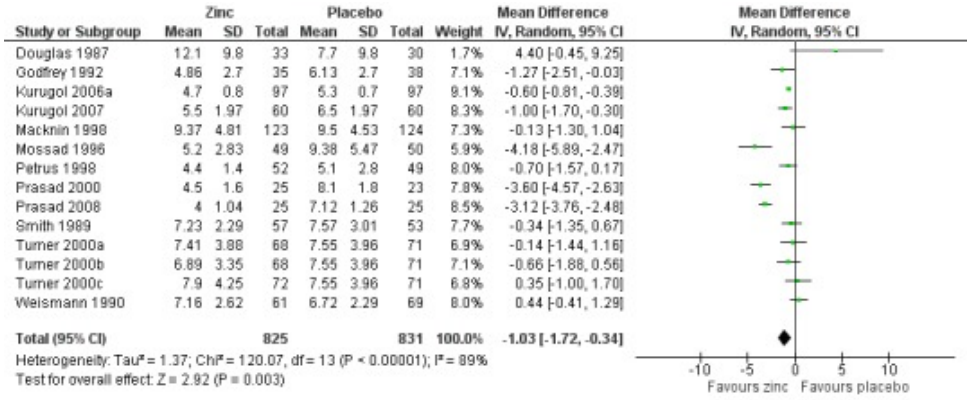


Figure 3

Forest plot of comparison: 1 Zinc versus placebo, outcome: 1.1 Duration of cold symptoms (in days).

FOI 26-3132 - Document 5

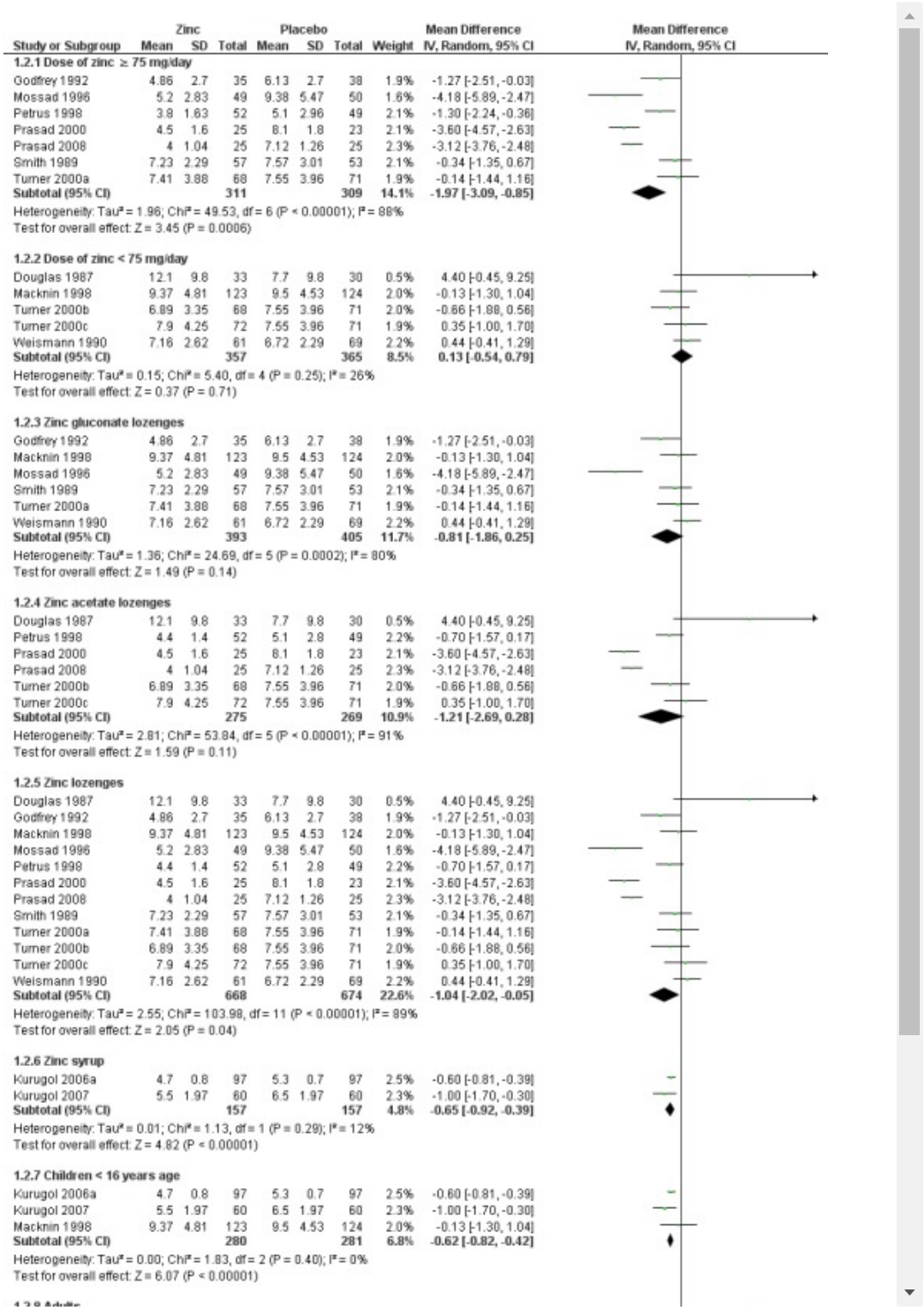
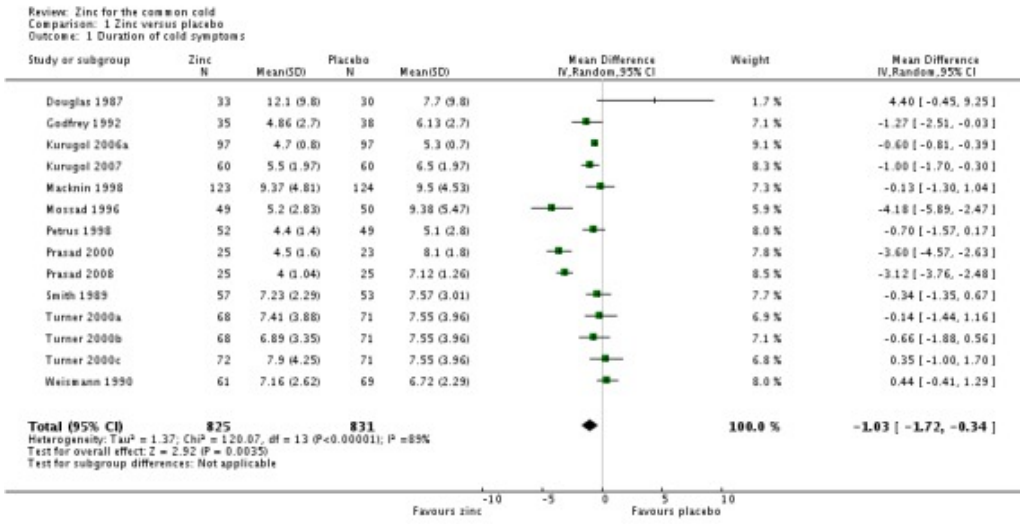


Figure 4

Forest plot of comparison: 1 Zinc versus placebo, outcome: 1.1 Duration of cold symptoms (in days).

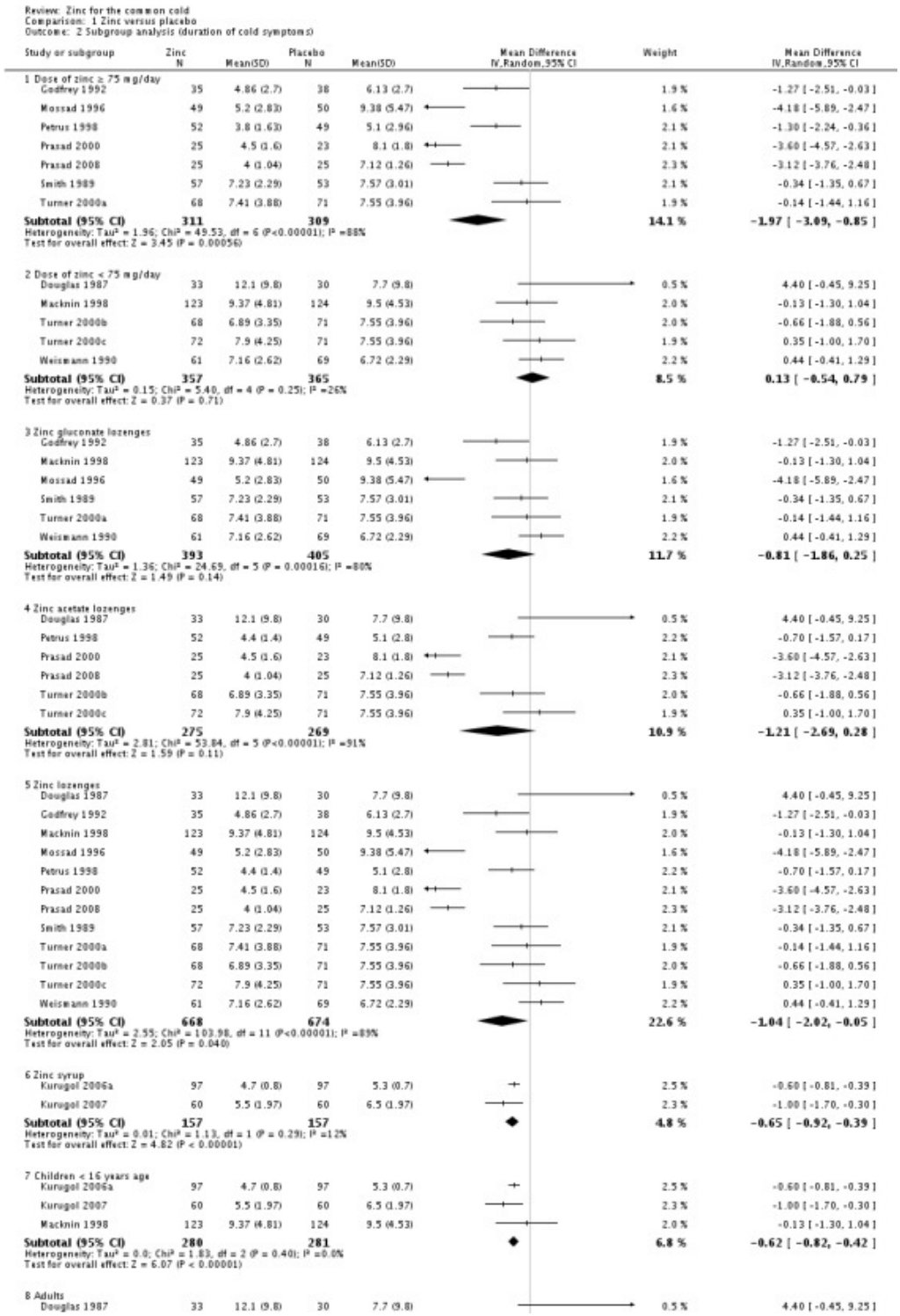
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Analysis 1.1

Comparison 1 Zinc versus placebo, Outcome 1 Duration of cold symptoms.

FOI 26-3132 - Document 5



Analysis 1.2

Comparison 1 Zinc versus placebo, Outcome 2 Subgroup analysis (duration of cold symptoms).

Seven trials (Godfrey 1992; Mossad 1996; Petrus 1998; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a) used zinc lozenges at a dose of ≥ 75 mg/day, and five trials (Douglas 1987; Macknin 1998; Turner 2000b; Turner 2000c; Weismann 1990) used a dose of < 75 mg/day. Pooled results from the trials using ≥ 75 mg/day of zinc showed a significant reduction in the duration (days) of common cold (MD -1.97, 95% CI -3.09 to -0.85) (P = 0.0006), whereas < 75 mg/day of zinc did not (MD 0.13, 95% CI -0.54 to 0.79) (P = 0.71). However, there was marked heterogeneity among the trials using ≥ 75 mg/day of zinc lozenges. Five trials (Godfrey 1992; Mossad 1996; Macknin 1998; Smith 1989; Turner

FOI 26-3132 - Document 5

2000a) used zinc gluconate lozenges, and six trials (Douglas 1987; Petrus 1998; Prasad 2000; Prasad 2008; Turner 2000b; Turner 2000c) used zinc acetate lozenges. Pooled result from the trials using either zinc gluconate (MD -0.81, 95% CI -1.86 to 0.25) (P = 0.14) or acetate lozenges (MD -1.21, 95% CI -2.69 to 0.28) (P = 0.11) did not show a reduction in the duration (days) of common cold. Again, there was marked heterogeneity among the trials. Twelve trials (Douglas 1987; Godfrey 1992; Mossad 1996; Macknin 1998; Petrus 1998; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990) used zinc lozenges, and two trials (Kurugol 2006a; Kurugol 2007) used zinc syrup formulations. Pooled result from the trials using either zinc lozenges (MD -1.04, 95% CI -2.02 to -0.05) (P = 0.04) or syrup formulations (MD -0.65, 95% CI -0.92 to -0.39) (P < 0.00001) showed a significant reduction in the duration (days) of common cold. There was marked heterogeneity among the trials using lozenges formulations. Eleven trials (Douglas 1987; Godfrey 1992; Mossad 1996; Petrus 1998; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990) studied the effect in adults, and three trials (Kurugol 2006a; Kurugol 2007; Macknin 1998) in children < 16 years age. Pooled results from the trials on adults (MD -1.12, 95% CI -2.17 to -0.06) (P = 0.04) or children (MD -0.62, 95% CI -0.82 to -0.42) (P < 0.00001) showed a significant reduction in the duration (days) of common cold. However, there was marked heterogeneity among the trials involving adult participants.

Severity of cold symptoms Thirteen studies measured the mean severity score of cold symptoms (Al-Nakib 1987; Douglas 1987; Godfrey 1992; Kurugol 2006a; Kurugol 2007; Petrus 1998; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990). Results from five studies (Kurugol 2006a; Kurugol 2007; Petrus 1998; Prasad 2000; Prasad 2008) including a total of 513 participants (Analysis 1.3; Figure 5) could be pooled. The difference was not significant between the intervention and control groups for reduction in the severity of cold symptoms (MD -1.06, 95% CI -2.36 to 0.23) (P = 0.11). In all but two studies, the intervention started within 24 hours of onset of symptoms. In the studies by Douglas 1987 and Kurugol 2007 the intervention started within 24 to 48 hours after the onset of symptoms. In the study by Godfrey 1992 the authors found a significant decrease in the severity of symptoms when treatment was administered within 24 hours, compared to treatment administration within 48 hours. In the trial by Al-Nakib 1987, the zinc group had a significantly lower mean daily clinical score than the placebo group; the difference in scores attaining statistical significance by day four and day five of treatment. However, in the study conducted by Douglas 1987, there were no significant differences between the two groups. Five studies (Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990) reported summed severity scores which could not be pooled. One study (Smith 1989) found a reduction in summed severity score in the zinc group, whereas others (Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990) did not. Again the dosages, formulations and time of administration of zinc differed among the studies. As insufficient data were available, we could only carry out subgroup analysis for lozenges versus syrup formulation (Analysis 1.4). Three trials (Petrus 1998; Prasad 2000; Prasad 2008) used lozenges and two trials (Kurugol 2006a; Kurugol 2007) used syrup formulations. Pooled results from the trials using either lozenges (MD -1.55, 95% CI -3.62 to 0.52) (P = 0.14) or syrup formulations (MD -0.27, 95% CI -1.51 to 0.97) (P = 0.67) did not show a reduction in the duration of the common cold. There was marked heterogeneity among the trials using the lozenge formulation.

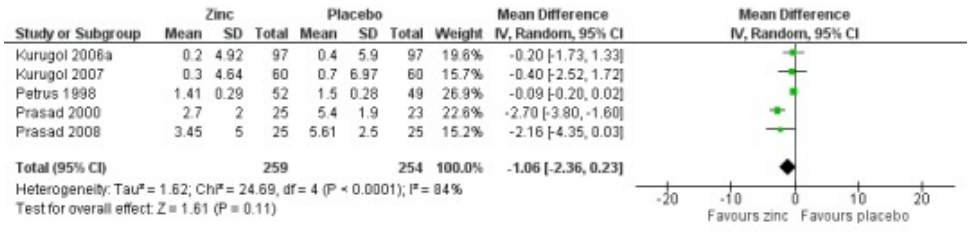
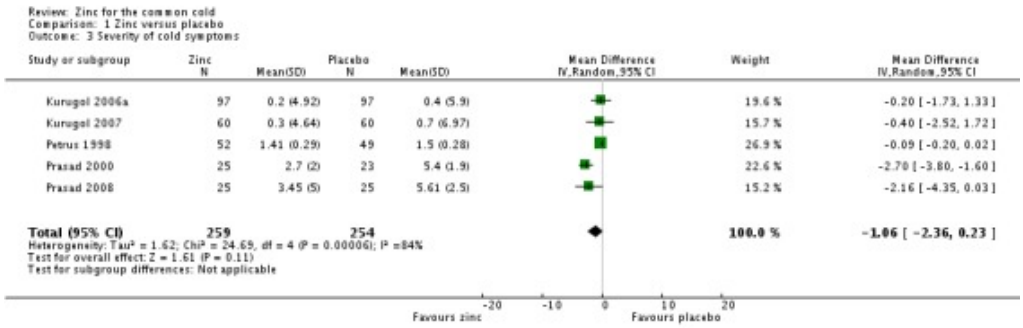


Figure 5

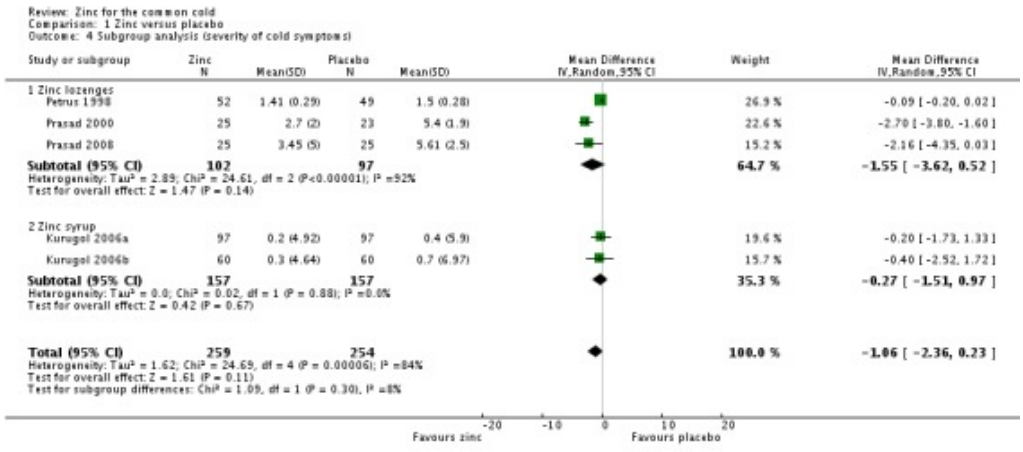
Forest plot of comparison: 1 Zinc versus placebo, outcome: 1.2 Severity of symptoms (score).

FOI 26-3132 - Document 5



Analysis 1.3

Comparison 1 Zinc versus placebo, Outcome 3 Severity of cold symptoms.



Analysis 1.4

Comparison 1 Zinc versus placebo, Outcome 4 Subgroup analysis (severity of cold symptoms).

(ii) Prophylactic effects of zinc

Incidence of common cold This was reported in two studies (Kurugol 2006b; Vakili 2009). The two studies used variable dose, formulation and duration of zinc. The follow-up periods of the two studies were different, therefore we based the calculation of the incidence rates on person-years. The person-time incidence rate is an appropriate measure of incidence when follow-up times are unequal (Rothman 1988). Incidence density is defined as the number of incident cases occurring in a susceptible population followed over a given time period; its units are therefore expressed as the number of cases per unit of person-time. The incidence density ratio is defined as the ratio of incidence density of an exposed group to that of an unexposed group. For each study, we calculated the incident rate ratio (IRR) of catching a cold in treated participants compared to the risk in control participants (Analysis 1.5; Figure 6). The IRR of developing a cold in subjects who received the intervention was 0.64 (95% CI 0.47 to 0.88), compared to participants in the control group (P = 0.006).

FOI 26-3132 - Document 5

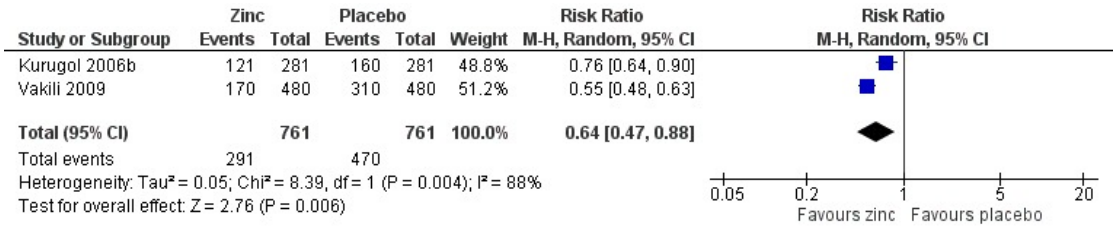
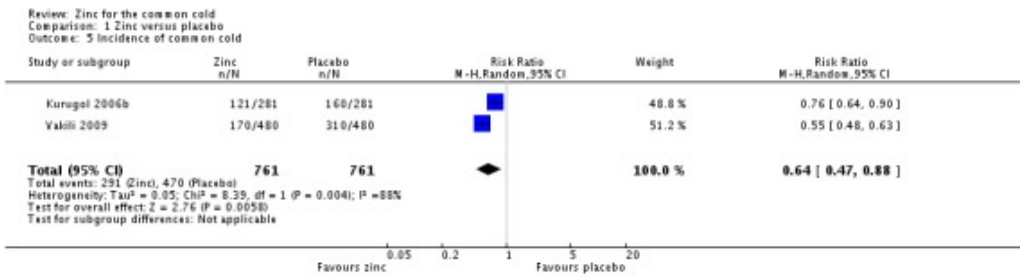


Figure 6

Forest plot of comparison: 1 Zinc versus placebo, outcome: 1.3 Incidence of common cold (IRR).



Analysis 1.5

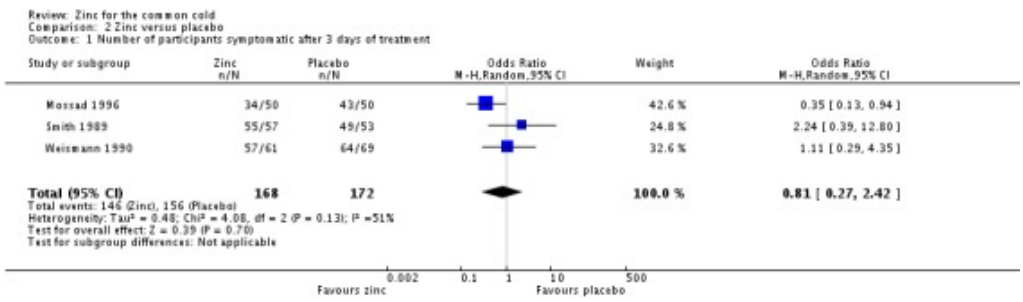
Comparison 1 Zinc versus placebo, Outcome 5 Incidence of common cold.

2. Secondary outcomes

(i) Therapeutic effects of zinc

Proportion of participants symptomatic after three, five or seven days of treatment

Proportion of participants symptomatic after three days of treatment Three studies (Mossad 1996; Smith 1989; Weismann 1990) included a total of 340 participants. There was no significant difference between the intervention and control group for the proportion of participants symptomatic after day three of treatment (odds ratio (OR) 0.81, 95% CI 0.27 to 2.42) (P = 0.7) (Analysis 2.1).



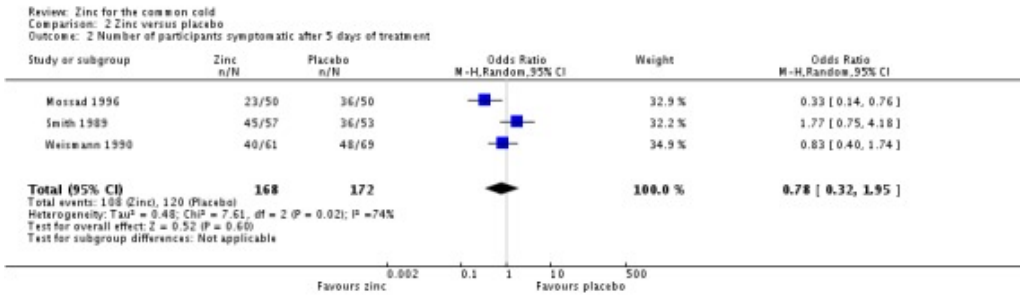
Analysis 2.1

Comparison 2 Zinc versus placebo, Outcome 1 Number of participants symptomatic after 3 days of treatment.

Proportion of participants symptomatic after five days of treatment

FOI 26-3132 - Document 5

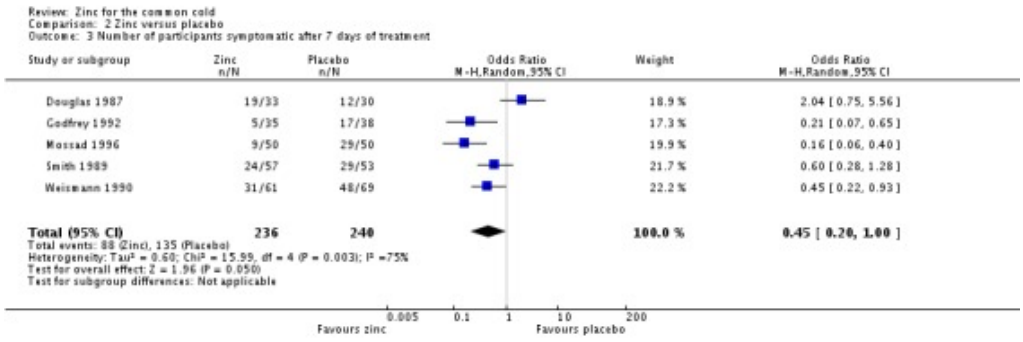
Three studies (Mossad 1996; Smith 1989; Weismann 1990) included a total of 340 participants. There was no significant difference between the intervention and control group for proportion of participants symptomatic after day five of treatment (OR 0.78, 95% CI 0.32 to 1.95) (P = 0.6) (Analysis 2.2).



Analysis 2.2

Comparison 2 Zinc versus placebo, Outcome 2 Number of participants symptomatic after 5 days of treatment.

Proportion of participants symptomatic after seven days of treatment Five studies (Douglas 1987; Godfrey 1992; Mossad 1996; Smith 1989; Weismann 1990) included a total of 476 participants. There was a significant difference between the intervention and control group for proportion of participants symptomatic after day seven of treatment (OR 0.45, 95% CI 0.20 to 1.00) (P = 0.05) (Analysis 2.3).



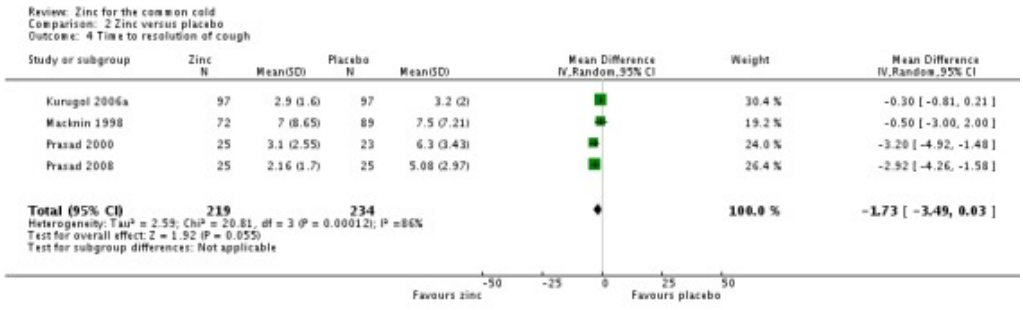
Analysis 2.3

Comparison 2 Zinc versus placebo, Outcome 3 Number of participants symptomatic after 7 days of treatment.

Time to resolution of individual cold symptoms This was reported in days in five studies.

Time to resolution of cough Four studies (Kurugol 2006a; Macknin 1998; Prasad 2000; Prasad 2008) included a total of 453 participants (intervention = 219, control = 234). The time taken for resolution of cough was significantly shorter in the intervention group (MD -1.73, 95% CI -3.49 to 0.03) (P = 0.05) (Analysis 2.4).

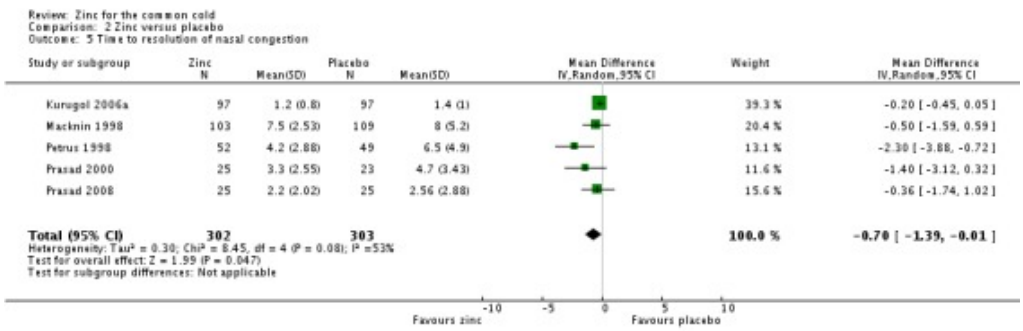
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Analysis 2.4

Comparison 2 Zinc versus placebo, Outcome 4 Time to resolution of cough.

Time to resolution of nasal congestion Five studies (Kurugol 2006a; Macknin 1998; Petrus 1998; Prasad 2000; Prasad 2008) included a total of 605 participants (intervention = 302, control = 303). The time taken for resolution of nasal congestion was significantly shorter in the intervention group (MD -0.7, 95% CI -1.39 to -0.01) (P = 0.02) (Analysis 2.5).

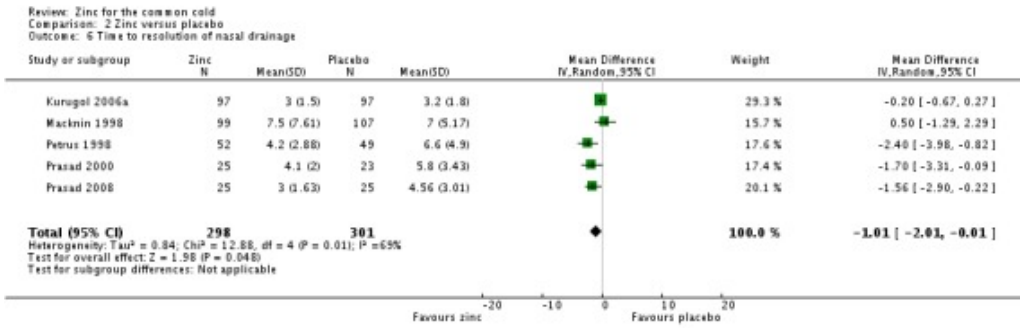


Analysis 2.5

Comparison 2 Zinc versus placebo, Outcome 5 Time to resolution of nasal congestion.

Time to resolution of nasal drainage Five studies (Kurugol 2006a; Macknin 1998; Petrus 1998; Prasad 2000; Prasad 2008) included a total of 599 participants (intervention = 298, control = 301). The time taken for resolution of nasal drainage was significantly shorter in the intervention group (MD -1.01, 95% CI -2.01 to -0.01) (P = 0.05) (Analysis 2.6).

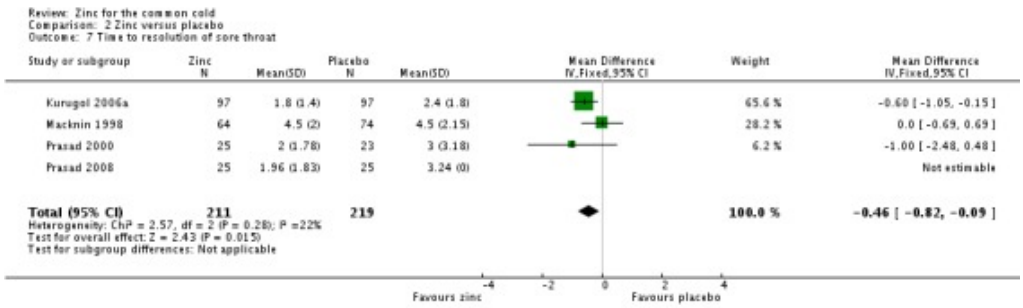
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Analysis 2.6

Comparison 2 Zinc versus placebo, Outcome 6 Time to resolution of nasal drainage.

Time to resolution of sore throat Four studies (Kurugol 2006a; Macknin 1998; Prasad 2000; Prasad 2008) included a total of 430 participants (intervention = 211, control = 219). The time taken for resolution of sore throat was significantly shorter in the intervention group (MD -0.46, 95% CI -0.82 to -0.09) (P = 0.02) (Analysis 2.7).



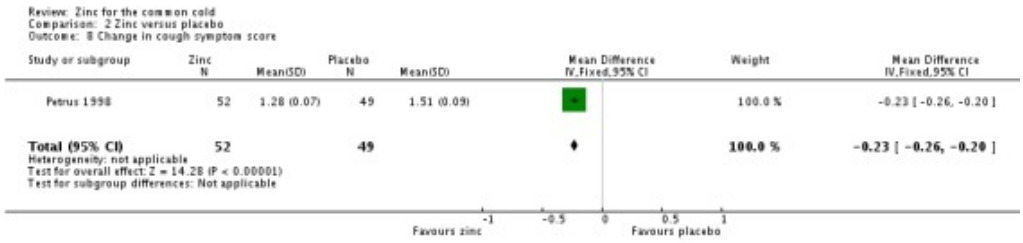
Analysis 2.7

Comparison 2 Zinc versus placebo, Outcome 7 Time to resolution of sore throat.

Change in individual severity symptom scores

Change in cough symptom score This was reported in two studies (Douglas 1987; Petrus 1998). In the study by Douglas 1987, a total of 63 treatment courses were evaluated (intervention = 33, control = 30) and the mean cough score (standard error of mean (SEM) not provided) was lower in the control group (6.3) than in the intervention group (10.6), which was not statistically significant (P = 0.2). In the study by Petrus 1998, a total of 101 participants were included and there was a significant decrease in the mean cough score in the intervention group (MD -0.23, 95% CI -0.26 to -0.2) (P < 0.00001) (Analysis 2.8).

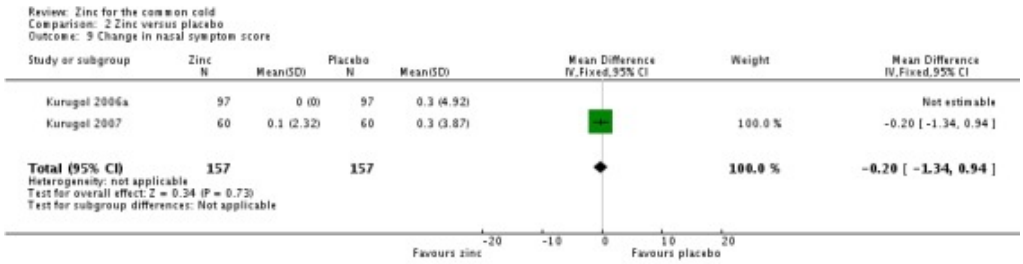
FOI 26-3132 - Document 5



Analysis 2.8

Comparison 2 Zinc versus placebo, Outcome 8 Change in cough symptom score.

Change in nasal symptom score This was reported in four studies (Douglas 1987; Kurugol 2006a; Kurugol 2007; Petrus 1998). In the study by Douglas 1987, a total of 63 treatment courses were evaluated and the mean nasal score (SEM not provided) was lower in the control group (9.8) than in the intervention group (11.7), which was not statistically significant (P = 0.5). In the study by Petrus 1998, a total of 101 participants were included and there was a decrease in the mean nasal score (not significant) in the intervention group (nasal congestion: placebo 1.43 ± 0.05, zinc 1.54 ± 0.08; nasal drainage: placebo 1.61 ± 0.07, zinc 1.45 ± 0.07). In the Kurugol 2006a and Kurugol 2007 studies a total of 314 participants were included and there was no difference between the two groups for the change in nasal symptom score (MD -0.2, 95% CI -1.34 to 0.94) (P = 0.73) (Analysis 2.9).



Analysis 2.9

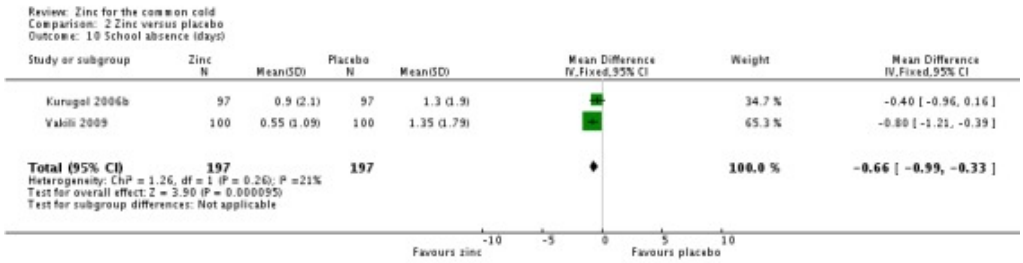
Comparison 2 Zinc versus placebo, Outcome 9 Change in nasal symptom score.

Change in throat symptom score This was reported in two studies (Douglas 1987; Petrus 1998). In one study (Douglas 1987), a total of 63 treatment courses were evaluated and the mean throat score (SEM not provided) was lower in the intervention group (6.1) than in the control group (6.2), which was not statistically significant (P = 0.96). In another study (Petrus 1998), a total of 101 participants were included and there was a decrease in the mean throat score (not significant) in the intervention group (sore throat: placebo 1.34 ± 0.11, zinc 1.26 ± 0.06; scratchy throat: placebo 1.53 ± 0.08, zinc 1.38 ± 0.1).

(ii) Prophylactic effects of zinc

School absence (days) Three trials reported this outcome. The pooled result from the two preventive trials (Kurugol 2006a; Vakili 2009) showed that zinc-supplemented children were absent for fewer days from school (MD -0.66, 95% CI -0.99 to -0.33) (P < 0.0001) (Analysis 2.10). In one of the therapeutic trials (Macknin 1998), children taking zinc were less likely to be absent than children taking placebo (OR 0.60, 95% CI 0.32 to 1.13) (P = 0.12), but the result was not significant.

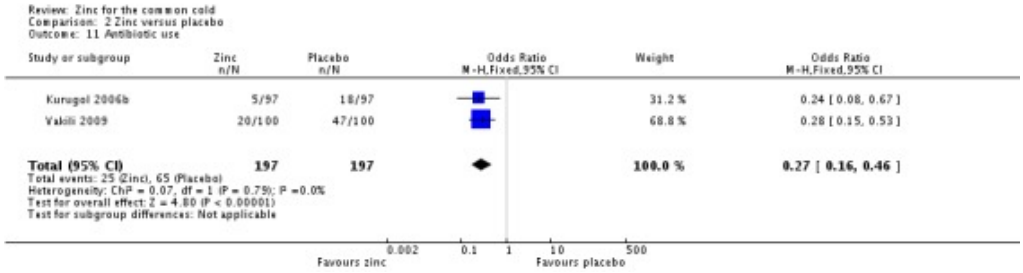
FOI 26-3132 - Document 5



Analysis 2.10

Comparison 2 Zinc versus placebo, Outcome 10 School absence (days).

Antibiotics use Two trials reported this outcome (Kurugol 2006b; Vakili 2009). The antibiotic prescription was more likely in placebo than in zinc-supplemented children (OR 0.27, 95% CI 0.16 to 0.46) (P < 0.00001) (Analysis 2.11).

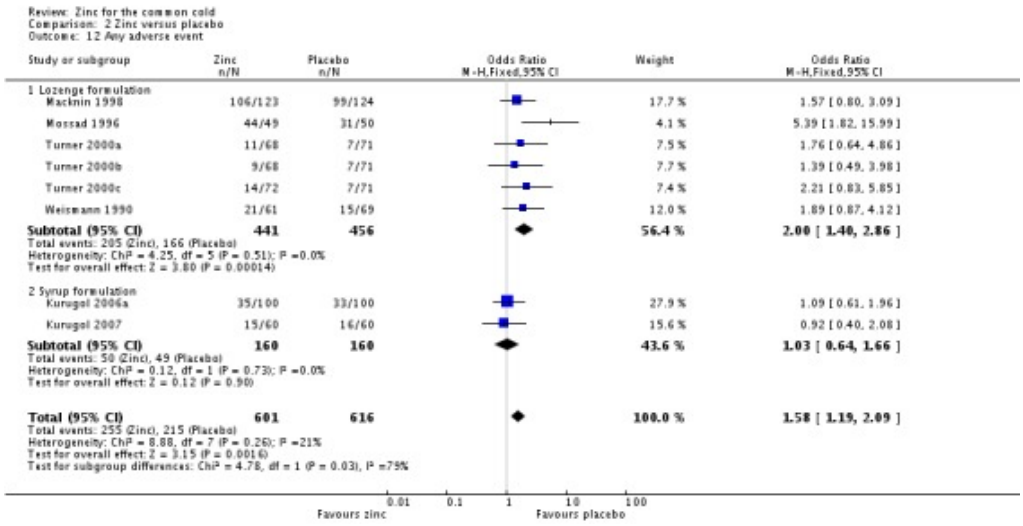


Analysis 2.11

Comparison 2 Zinc versus placebo, Outcome 11 Antibiotic use.

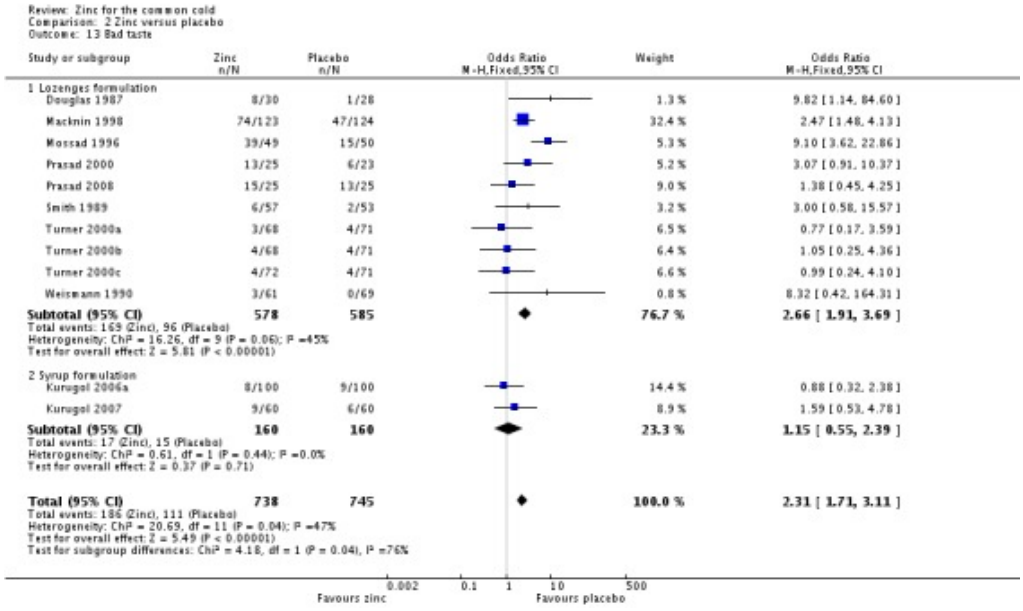
(iii) Adverse events Thirteen trials (Douglas 1987; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Macknin 1998; Mossad 1996; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Weismann 1990) reported any or individual adverse events. The incidence of any adverse event was higher in the zinc group (OR 1.58, 95% CI 1.19 to 2.09) (P = 0.002) than in the placebo group. Among the zinc group, the lozenges formulation (OR 2.00, 95% CI 1.40 to 2.86) (P = 0.0001) was more like to produce any adverse events than the syrup formulation (OR 1.03, 95% CI 0.64 to 1.66) (P = 0.9) (Analysis 2.12). Among individual events, bad taste (OR 2.31, 95% CI 1.71 to 3.11) (P < 0.00001) (Analysis 2.13) and nausea (OR 2.15, 95% CI 1.44 to 3.23) (P = 0.002) (Analysis 2.14) had a higher incidence in the zinc group. Among the zinc group, the lozenges formulation (OR 2.66, 95% CI 1.91 to 3.69) (P < 0.00001) was more like to produce bad taste than the syrup formulation (OR 1.15, 95% CI 0.55 to 2.39) (P = 0.71). There was no significant difference between the two groups in the incidence of constipation (P = 0.17) (Analysis 2.15), diarrhoea (P = 0.08) (Analysis 2.16), abdominal pain (P = 0.25) (Analysis 2.17), dry mouth (P = 0.09) (Analysis 2.18) and oral irritation (P = 0.50) (Analysis 2.19).

FOI 26-3132 - Document 5



Analysis 2.12

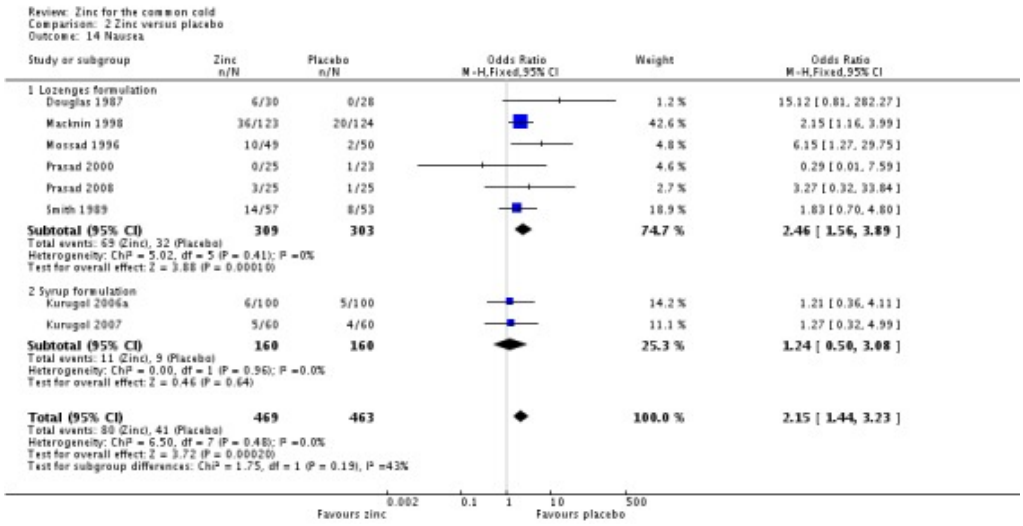
Comparison 2 Zinc versus placebo, Outcome 12 Any adverse event.



Analysis 2.13

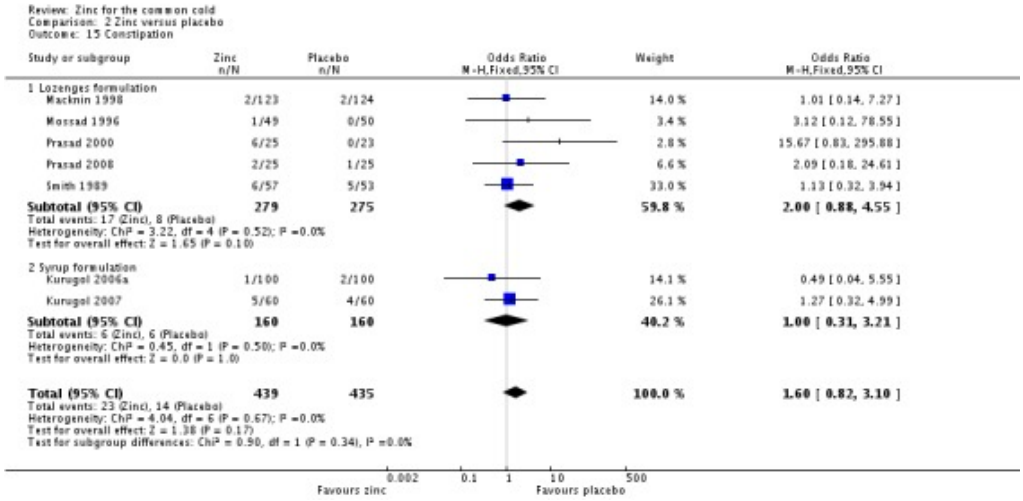
Comparison 2 Zinc versus placebo, Outcome 13 Bad taste.

FOI 26-3132 - Document 5



Analysis 2.14

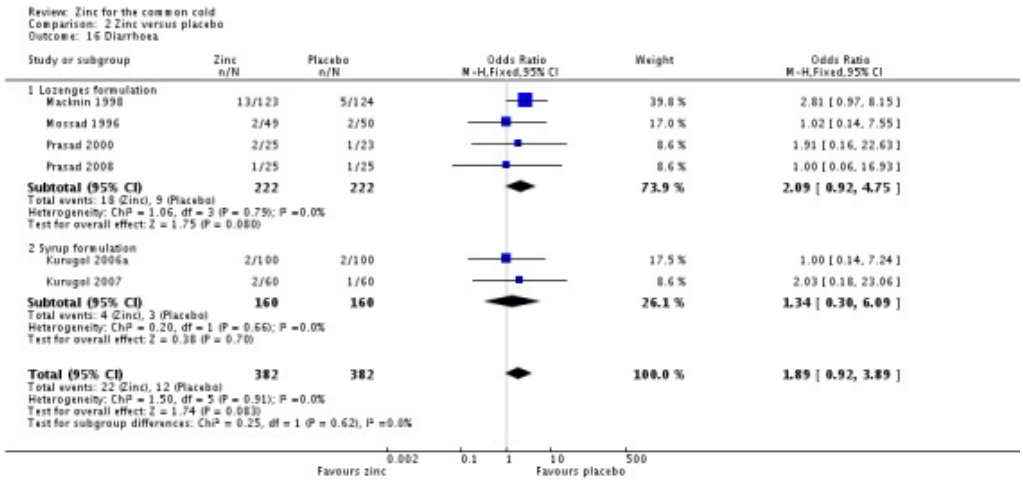
Comparison 2 Zinc versus placebo, Outcome 14 Nausea.



Analysis 2.15

Comparison 2 Zinc versus placebo, Outcome 15 Constipation.

FOI 26-3132 - Document 5



Analysis 2.16

Comparison 2 Zinc versus placebo, Outcome 16 Diarrhoea.

Analysis 2.17

Comparison 2 Zinc versus placebo, Outcome 17 Abdominal pain.

Analysis 2.18

Comparison 2 Zinc versus placebo, Outcome 18 Dry mouth.

Analysis 2.19

Comparison 2 Zinc versus placebo, Outcome 19 Mouth irritation.

(iv) Publication bias To assess whether there was a bias in the published literature, we plotted the effect size of each trial versus variance for one of the primary outcome (duration of common cold). The funnel plot generated here shows that most of the precise studies (towards the top of the plots) have effect sizes which are either zero or very close to it (Analysis 1.1). One explanation for such asymmetry might be publication bias.

Discussion

The discussion is divided into two parts: the first will discuss important methodological issues that have emerged from research in this area, and the second part will discuss the results obtained and their clinical significance.

FOI 26-3132 - Document 5

Part 1: methodology

Since Eby's trial in 1984 (Eby 1984), many trials have investigated whether zinc is efficacious in the treatment or prevention of the common cold. Among these 18 trials were included in this 2013 review. We rated the methodological quality of the included trials as good, with two trials excluded because of poor quality. Eby's trial realised a number of limitations which raised concerns regarding the validity of the results. Treatment blinding in the trial has been questioned as zinc lozenges were found to be unpalatable, distorted the taste of participants and caused a higher incidence of side effects. In addition, investigators relied solely on the subjective assessment of cold symptoms; laboratory confirmation of viral infection was not conducted and analyses were only conducted on a subgroup of those originally enrolled in the trial. Eby's trial was nevertheless instructive and highlighted a number of methodological issues.

Like Eby's trial, most trials have relied on community-acquired infections. However, five trials recruited participants from volunteers experimentally inoculated with human rhinovirus (Al-Nakib 1987; Farr 1987a; Turner 2000a; Turner 2000b; Turner 2000c). While high rates of infection with human rhinovirus were attained in the later trials and most participants experienced cold symptoms, in trials relying on community-acquired infection, the infecting agent and the infection rates were generally not determined.

In trials relying on community-acquired infection, investigators relied on trial participants or family members to assess the incidence and severity of cold symptoms. Though in most of the trials information was generally provided on how compliance with the recording of symptoms was assessed, objective periodic assessments of the clinical severity of respiratory symptoms were not conducted. In the trials conducted by Al-Nakib 1987, Farr 1987a, Macknin 1998, Kurugol 2006a, Kurugol 2006b, Kurugol 2007 and Turner 2000a, Turner 2000b and Turner 2000c, symptoms were assessed by trial personnel thus providing some assurance as to the validity of clinical severity scores and estimates based on such scores. Assessment of response to treatment also depended on objective measurements such as nasal mucus weight or tissue counts, which was measured in one study (Al-Nakib 1987) and the authors found that zinc gluconate reduced both of these parameters. However, as in most studies children were involved, this was not practical.

Research by Farr 1987b suggested that in most trials the size of the placebo-blinding study used to determine whether zinc and placebo lozenges were indistinguishable was not sufficiently large to detect a significant difference. In their efforts to find a suitable matching placebo lozenge, Farr showed that a false negative result may occur if a small subject population (i.e. fewer than 20) is used. Given that placebo-blinding studies were only conducted in six of the 18 trials, and with the exception of three trials (Farr 1987a; Prasad 2000; Prasad 2008) the size of the placebo-matching studies in two of the remaining three trials (no information was provided on the size of placebo study conducted by Weismann 1990) ranged from eight to 20, the adequacy of blinding in most trials is questioned. Zinc-treated participants also experienced higher incidences of side effects, complaints or both, and in seven trials zinc-treated participants complained of altered, bad or unpalatable taste, which suggests that zinc lozenges were distinct from placebo lozenges and, in this respect, blinding may have been compromised. However, the increased incidence of bad taste and nausea found by Mossad 1996; constipation and mouth dryness found by Prasad 2000; and bad taste, nausea, mouth, tongue or throat discomfort and diarrhoea found by Macknin 1998, may have been related to the use of different ligands (gluconate, acetate) rather than to zinc itself.

Much of the controversy surrounding the use of zinc lozenges in the treatment of the common cold has concerned whether formulations used in trials showing no benefit failed to release sufficient zinc ions to be effective. It has been hypothesised that there is a direct correlation between reductions in the duration of common cold symptoms and the daily dosage of all positively charged zinc species released from lozenges at physiologic pH (Eby 1995). The reanalysis of 10 double-blind, placebo-controlled zinc trials by solution chemistry methods showed a significant correlation between total daily dosages of positively charged zinc species and a reduction in the mean duration of common colds (Eby 2004). Zinc gluconate and zinc acetate have very low chemical stability and mainly release positively charged zinc ions in aqueous solutions at physiologic pH, but stronger complexes do not (Eby 2004). Adding a strong zinc-binding ligand, such as glycine, citric acid, tartaric acid, mannitol and sorbitol, to a solution

FOI 26-3132 - Document 5

containing a zinc complex that is weakly bonded results in the sequestration of zinc to the stronger ligand, reducing or eliminating the benefits of zinc lozenges (Eby 2004). The extent to which the zinc ion was released from formulations reporting no benefit is not known. However, experimental evidence suggests that in saliva the ionisation of zinc to free zinc for some formulations may have been completely (Zarembo 1992) or partially (Farr 1988) suppressed. A formulation developed by Godfrey (Godfrey 1992) that incorporates glycine has been shown to release more than 50% of the zinc from zinc gluconate as the zinc ion. Results from trials conducted by Godfrey (Godfrey 1992) and Mossad (Mossad 1996) suggest this formulation reduced the duration and severity of respiratory symptoms; whereas the trials conducted by Macknin (Macknin 1998) and Turner (Turner 2000a; Turner 2000b; Turner 2000c) suggest no effect of this formulation. The placebo-matching was inadequate; consequently the adequacy of blinding in all these trials is questioned. Hatch et al (Hatch 1987) reported that zinc acetate releases essentially 100% of its zinc as Zn^{2+} ions at a physiological pH. Thus, use of zinc lozenges may be advantageous. Results from trials conducted by Petrus (Petrus 1998) and Prasad (Prasad 2000; Prasad 2008) suggest this formulation reduced the duration and severity of respiratory symptoms. These studies used compressed lozenges designed by George Eby that were identical in composition. In addition to zinc acetate, they contained directly compressible (agglomerated) dextrose as the tablet base, glycerol monostearate (2.5% of tablet weight) as the tablet lubricant, stevia for added sweetness, and peppermint oil for flavour, with the composition compressed to near maximal hardness for slowest dissolution. These ingredients were specifically chosen because they do not react with ionic zinc. The trials conducted by Douglas (Douglas 1987) and Turner (Turner 2000a; Turner 2000b; Turner 2000c) using zinc acetate suggest no effect of this formulation. Among these, the placebo-matching and blinding were stated to be adequate in all except the trial by Turner. Three trials (Kurugol 2006a; Kurugol 2006b; Kurugol 2007) used syrup preparation of zinc (zinc sulphate) and found reduced duration and severity of respiratory symptoms without any increase in adverse effects in the zinc group. Placebo-matching and adequacy of blinding was not stated in these trials. Another trial (Vakili 2009) used tablet preparation (zinc sulphate) and found decreased incidence, fewer school absences, less antibiotic administration and no adverse effects in zinc-supplemented children. Again placebo-matching and adequacy of blinding was not stated in this trial.

The toxicology of zinc has been well characterised. The potential for elevated blood levels of zinc to disrupt copper metabolism and other nutrients preclude its long-term use in the treatment of the common cold (Pfeiffer 1980). Doses higher than 150 mg/day have also been associated with adverse effects (Chandra 1984). In addition, the higher incidence of side effects in zinc-treated participants will most likely limit the usefulness of zinc in the treatment of common cold symptoms. In 14 trials (Douglas 1987; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Macknin 1998; Mossad 1996; Prasad 2000; Prasad 2008; Smith 1989; Turner 2000a; Turner 2000b; Turner 2000c; Vakili 2009; Weismann 1990) included for reporting of any or individual adverse events, the overall adverse events were higher in the intervention than in the control group, except in one trial (Vakili 2009).

Viral studies were performed in nine trials (Al-Nakib 1987; Douglas 1987; Farr 1987a; Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Turner 2000a; Turner 2000b; Turner 2000c). While in-vitro studies suggest zinc inhibits viral replication and the concentration of zinc in saliva should be sufficient to induce such an effect, six trials (Al-Nakib 1987; Douglas 1987; Farr 1987a; Turner 2000a; Turner 2000b; Turner 2000c) found no effect of zinc on incidence or shedding of rhinovirus by study participants. In relation to this, trials by Farr and Douglas found no effect of zinc in the treatment of cold, while Al-Nakib found reduction in the clinical symptom score. From the trial by Al-Nakib, it might be suggested that medication may have had an effect on signs and symptoms of the colds rather than on virus replication. If this is the case, it would be interesting to know whether zinc would also have the same effect on corona virus colds or, indeed, on colds caused by other respiratory viruses. This may be the future area of research in zinc and common cold trials. Trials by Kurugol (Kurugol 2006a; Kurugol 2006b; Kurugol 2007) did not study the effect of zinc on rhinovirus cold; rather they excluded colds due to influenza viruses from analysis and found that zinc is effective in the treatment of the common cold.

Part 2: results

FOI 26-3132 - Document 5

Although most investigators required participants to record the clinical severity of symptoms each day and used similar scales against which to rate symptom severity (symptoms were rated as none, mild, moderate or severe), there was little commonality in summary estimates used by investigators to describe the duration, incidence and severity of respiratory symptoms. Consequently for some outcomes it was not possible to pool the results from all the trials that reported the same outcome. In addition, there was insufficient detail provided in most published papers to determine whether trials used similar criteria for rating the severity of each symptom and therefore it was not possible to standardise clinical severity scores across all trials.

Among the trials reporting the duration of cold symptoms, results could be pooled from 14 trials. Intake of zinc lozenges or syrup was associated with a significant reduction in the duration of common cold, but there was marked heterogeneity among the included trials. Among these, two trials (Kurugol 2006a; Kurugol 2007) used syrup preparation at a daily dose of 30 mg, whereas other trials used lozenge preparation at a daily dose varying from 30 mg to 190 mg. In a subgroup analysis, the results were significant for the dose of zinc lozenges ≥ 75 mg/day, any formulation of zinc (lozenges or syrup) and any age group (children or adult). Results were not significant for any formulations of zinc lozenges (gluconate or acetate). However, the heterogeneity persisted for all these subgroups except syrup formulation of zinc. Among the two trials using syrup preparation, one trial (Kurugol 2007) found no significant effect, and the other found significant reduction of the duration of common cold. There has been speculation regarding how zinc cures common cold. The most biologically plausible hypothesis, as well as experimental, was by Eby (Eby 2010; Eby 2012). As described by him, the mouth–nose act as a biologically closed electric circuit (BCEC). It moves electrons from the nose into the mouth and, in response to the electron flow, it moves positively charged metal ions, such as ionic zinc, from the mouth into the nose. This BCEC does not transport neutrally or negatively charged zinc into the nose; so the lozenges made with non-positively charged zinc are supposed not to work in common cold therapy. Considering these properties, the observation that zinc lozenges releasing positively charged ionic zinc shorten colds in a dose–response manner can be seen in a more plausible light. This is also supported by finding of usefulness of ≥ 75 mg/day of zinc lozenges in reducing the duration of common cold in the present review. The BCEC also explains why patients with colds and a history of allergy responded so much faster than patients with colds and no history of allergy, as shown by Petrus (Petrus 1998). Orally ingested forms such as tablets, liquids and syrups do not have these properties and therefore, according to Eby's research, are not effective therapy for the common cold (Eby 2012).

Among 13 trials measuring the severity score of cold symptoms, results from five trials (Kurugol 2006a; Kurugol 2007; Petrus 1998; Prasad 2000; Prasad 2008) could be pooled. There was a significant difference between the intervention and control group for reduction in the severity of cold symptoms. The trials using syrup preparation used a daily dose of 30 mg, whereas the trials using lozenge preparation used daily doses varying from 30 mg to 276 mg. Al-Nakib (Al-Nakib 1987) and Douglas (Douglas 1987) provided estimates of mean clinical scores. However, estimates were not directly comparable. In the trial by Al-Nakib, the zinc group had a significantly lower mean daily clinical score than the placebo group, with the difference in scores attaining statistical significance on days four and five. However, in the trial conducted by Douglas, there were no statistically significant differences between zinc and placebo groups with respect to mean nasal, throat and cough scores. Results of the trial conducted by Godfrey (Godfrey 1992) suggested treatment with zinc reduced the frequency and severity of cold symptoms, which was noticeable by day five and significant by day seven. In the trial conducted by Turner (Turner 2000a; Turner 2000b; Turner 2000c) none of the zinc preparations (neither gluconate nor acetate) had any significant effect on the severity of common cold symptoms in the first three days of treatment, which might be related to the lower dose of zinc used in this trial. Among four trials (Douglas 1987; Kurugol 2006a; Kurugol 2007; Petrus 1998) measuring individual symptom scores there was a significant reduction in the cough score, with nasal and throat score being variably affected. In the trial conducted by Smith 1989, the zinc group had lower symptom severity scores on days four to seven of treatment which was statistically significant (P = 0.02); but in the trial conducted by Weismann 1990, no statistically significant differences between the two groups were found by day six of treatment (P = 0.14).

FOI 26-3132 - Document 5

Among the two preventive trials measuring the incidence of the common cold (Kurugol 2006b; Vakili 2009), the incidence rate ratio (IRR) of developing a cold in participants who received the intervention was lower than in the placebo group. There was marked heterogeneity, therefore we used a random-effects model for analysing this outcome. The second trial, though a randomised controlled trial (RCT), was not of good methodological quality, but this was included in the analysis as it included a large number of participants. Even after excluding this trial from analysis, the result still favoured zinc supplementation. The first trial used zinc sulfate syrup at a daily dose of 15 mg for seven months, whereas the second trial used zinc sulfate tablet at a daily dose of 10 mg for five months.

The proportion of participants who were asymptomatic after three, five and seven days of treatment was reported in the trials conducted by Mossad 1996, Weismann 1990 and Smith 1989, and the proportion asymptomatic after seven days of treatment was reported in all but the trials conducted by Farr 1987a and Al-Nakib 1987. Analyses were conducted on an intention-to-treat (ITT) basis. In the trial conducted by Mossad (Mossad 1996) participants were less likely to have cold symptoms after three and five days of treatment in the zinc-treated group. The odds ratios (ORs) for days three and five were 0.37 (95% confidence interval (CI) 0.14 to 0.92) and 0.35 (95% CI 0.16 to 0.76), respectively. In the trials conducted by Weismann 1990 and Smith 1989, the proportion of participants asymptomatic after three and five days in the zinc and placebo groups was similar. The test for heterogeneity attained statistical significance for day five, but not day three and consequently a combined OR for day five is not appropriate. The combined OR for day three was not significant 0.97 (95% CI 0.62 to 1.5). In three trials (Godfrey 1992; Mossad 1996; Weismann 1990), fewer participants in the zinc group had cold symptoms after seven days. The pooled result (OR 0.53, 95% CI 0.38 to 0.75) obtained from five trials (Douglas 1987; Godfrey 1992; Mossad 1996; Smith 1989; Weismann 1990) indicated fewer participants in the zinc group had cold symptoms after seven days of treatment. However, the test for heterogeneity was statistically significant.

In six trials (Godfrey 1992; Kurugol 2006a; Mossad 1996; Petrus 1998; Prasad 2000; Prasad 2008) with similar study designs, methodologies and efficacy assessments, zinc was found to be effective in reducing the duration and severity of common cold symptoms in healthy children and adults, when it was administered within 24 hours of the onset of symptoms. In another trial (Kurugol 2007) with similar study design, methodology and efficacy assessments, zinc was found to be effective in reducing the severity of common cold symptoms in healthy children (without any change in duration), when it was administered within 24 to 48 hours of the onset of symptoms. In the trial by Godfrey 1992, the authors found a significant decrease in the duration and severity of symptoms when treatment was administered within 24 hours, compared to treatment administration within 48 hours. So, if treatment with zinc is to be used for common cold, it should ideally commence within 24 hours of onset of symptoms.

There are a number of potential sources of heterogeneity in results obtained from trials included in this review. Most trials relied on community-acquired infections in which the infecting agent was not identified and as such different agents may have been involved which may have differed in their sensitivity to zinc. The amount of zinc taken each day by participants varied largely across the trials, and given that some formulations released less zinc ion than others the effective dose of zinc across trials was variable. Blinding of treatment may not have been adequately controlled in some trials, thereby increasing the potential for performance and detection bias to occur. The time from onset of cold symptoms to commencement of treatment ranged from one to three days. Given the beneficial effects noted in trials commencing treatment with zinc within 24 hours, the results from all the trials may not be comparable. Last but not the least is the fact that the lifestyle of the study population in all the trials was different and the results might have been affected to some degree by this factor.

Summary of main results

Studies reporting duration and severity of cold symptoms suggest that the intake of zinc is associated with a significant reduction in the overall duration and severity of common cold symptoms. A higher proportion of participants became asymptomatic by day seven of treatment with zinc. Duration of individual cold symptoms was also significantly reduced in the zinc group, though the individual symptom severity scores were not significantly affected by the intake of zinc. Zinc supplementation led

FOI 26-3132 - Document 5

to reduction in the incidence of common cold, decreased school absence and decreased the risk of antibiotic use when used for at least five months. The incidence of adverse events was significantly higher in the zinc group with the syrup preparation being better tolerated than lozenges.

Overall completeness and applicability of evidence

The trials included in the analysis involved healthy children and adults of all ages (except infants) and both sexes. All except four trials (Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Vakili 2009) were conducted in upper-middle and high-income countries, where zinc deficiency is uncommon (including in young children). Out of four trials (Kurugol 2006a; Kurugol 2006b; Kurugol 2007; Vakili 2009), three were conducted in Turkey and one in Iran, which are low-income countries. These later trials found beneficial effects of zinc when used either in syrup or tablet (not lozenges) form. So, any beneficial effect of zinc lozenges might not be directly extrapolated to children and adults of low-income countries. In all the included trials, the main weakness of the data is that most trials, when presenting data, did not differentiate between cold due to rhinovirus and other viruses (as viral studies were not conducted in most of these trials). So it is unclear whether zinc helps those with rhinoviral cold or even cold due to other viruses. However, as rhinovirus is the most common aetiological agent of the common cold all over the world (in both low-income and high-income countries), it may be predicted that zinc lozenges prepared in the above mentioned formulations might also help people living in low-income countries.

Quality of the evidence

The trial evidence included is generally of good quality, with a low risk of bias. All the studies were blinded, but placebo-blinding was adequately described in only six trials. In 12 trials, zinc-treated participants complained of altered, bad or unpalatable taste which suggests that zinc lozenges were distinct from placebo lozenges and, in this respect, blinding may have been compromised. Allocation concealment was adequate in nine studies and unclear in nine studies. Thirteen trials reported a low rate of loss to follow-up, whereas three trials did not mention any loss to follow-up. This suggests that the studies were of good quality. For all the outcomes, there was more than one study reporting the individual outcome. The majority were carefully conducted community trials, with active mechanisms to promote adherence to the intervention and both active and passive case finding. Last and most importantly, as there was a high degree of heterogeneity among the trials, the result should be interpreted with caution.

Potential biases in the review process

All the included trials, as expected, measured the effect of zinc on the common cold. There was therefore the potential to miss trials which may have measured common cold as upper respiratory tract infection in secondary outcomes which were less publicised or less well indexed within the electronic databases. We tried to avoid this by conducting a wide search and assessing the relevance of each paper identified in that search carefully. We extracted data from survival curves or t/P values given in studies that did not provide original data, which might have overestimated or underestimated the effect size. Lastly, publication bias cannot be ruled out as shown by funnel plot asymmetry.

Agreements and disagreements with other studies or reviews

The important changes in this updated review in comparison to the previous version (Marshall 1999) include the following.

1. Intake of zinc is associated with a significant reduction in the duration and severity of common cold symptoms.
2. Duration of individual cold symptoms was also significantly reduced in the zinc group.
3. The syrup and tablet preparation of zinc is better tolerated than lozenges.
4. Zinc supplementation reduces incidence, school absence and prescription of antibiotics in children with the common cold.

FOI 26-3132 - Document 5

In the review conducted by Marshall (Marshall 1999) the included studies had missing information, due to which it was not possible to pool the results across the studies. For example, although most investigators required participants to record the clinical severity of symptoms each day and used similar scales against which to rate symptom severity (symptoms were rated as none, mild, moderate or severe), there was little commonality in the summary estimates used by investigators to describe the duration, incidence and severity of respiratory symptoms. It was therefore only possible to determine the proportion of participants who were asymptomatic after three and five days of treatment for three trials and after seven days of treatment for five trials. Except for one study (Godfrey 1992), duration of symptoms was not reported in the rest of the studies. Therefore, again it was not possible to pool the results for this outcome.

In the revisit of their previous meta-analysis, Jackson (Jackson 2000) found statistically significant heterogeneity between the zinc trials. They calculated a pooled estimate of the zinc gluconate lozenges in colds using the random-effects model of DerSimonian and Laird from eight trials. The summary OR for the presence of "any cold symptoms" at seven days was 0.52 (95% CI 0.25 to 1.2). They concluded that despite numerous randomised trials, the evidence for the effectiveness of zinc lozenges in reducing the duration of common colds is still lacking. Although, according to them, some of the negative results might be explained by low zinc ion availability, they did not examine the issue. Moreover, they have not looked for the effect of any other formulations, as well as high or low doses of zinc.

A review published in 2004 by Hulisz 2004, which was an overview of published articles through MEDLINE (1980 to 2003), concluded that zinc effectively reduces the duration and severity of common cold symptoms when administered within 24 hours of the onset of symptoms. The author also had some concerns regarding the clinical tests of zinc for the treatment of common colds being inconsistent, primarily because of study design, blinding and lozenge contents; early formulations of lozenges being unpalatable with a higher incidence of side effects.

In a systematic review of trials published between 1966 and 2006, Caruso 2007 used 11 features of experimental design affecting signal quality, chance, bias and blinding to evaluate 14 placebo-controlled trials. They gave one point for each of the 11 quality items if it was satisfied, after which only four trials obtained the maximum of 11 points. Based on this, they suggested that the positive results in the zinc trials might be explained by methodological flaws. However, the use of scales for assessing quality or risk of bias is strongly discouraged in Cochrane systematic reviews. Moreover, such an approach, though simple, is not supported by evidence (Higgins 2011).

In another systematic review by Hemilä (Hemilä 2011), the author included 13 placebo-controlled comparisons. Pooling of trials using a total daily zinc dose of less than 75 mg found no effect, but those using zinc acetate in daily doses of over 75 mg found a 42% reduction, and those using zinc salts other than acetate in daily doses of over 75 mg found a 20% reduction in the duration of colds. The author concluded that there is strong evidence that the zinc lozenge effect on common cold duration is heterogeneous so that benefit is observed with high doses of zinc but not with low doses. However, there were no descriptions of other formulations of zinc (syrup or tablet).

In the latest systematic review (Science 2012), the authors included 17 trials. Compared with patients given placebo, those receiving zinc had a shorter duration of cold symptoms (mean difference (MD) -1.65 days, 95% CI -2.50 to -0.81). However, heterogeneity was high. Zinc shortened the duration of cold symptoms in adults but no significant effect was seen among children. In contrast to this, we found zinc to be effective both in children (< 16 years age) and adults. The reduction in the duration of cold symptoms was greater with high doses of ionic zinc (MD -2.75, 95% CI -3.89 to -1.60) than with lower doses (MD -0.84, 95% CI -1.50 to -0.18). However, we found that only zinc lozenges containing high dose (≥ 75 mg/day) reduced the cold duration significantly.

Authors' conclusions

Evidence shows that zinc is beneficial for the common cold in healthy children and adults living in high-income countries. Pooled results from the trials showed that zinc reduced the duration (not the severity) of common cold symptoms when used therapeutically. However, the effect of zinc

FOI 26-3132 - Document 5

lozenges on common cold duration is heterogeneous so that benefit is observed with high doses but not with low doses of zinc. The effects of zinc lozenges should be further studied to determine the optimal lozenge compositions and treatment strategies. Zinc also reduced the incidence of the common cold, school absence and antibiotic use in healthy children when used prophylactically, although antibiotics are not required for the common cold. All the studies included healthy participants; we could not find any evidence regarding use of zinc in participants at increased risk of developing the common cold. As the zinc lozenges formulation has been widely studied, and there is a significant reduction in the duration (days) of cold at a dose of ≥ 75 mg/day, for those considering using zinc it would be best to use it at this dose throughout the cold. Regarding prophylactic zinc supplementation, currently no firm recommendation can be made because of insufficient data. When using zinc lozenges one should be aware of the side effects.

Morbidity associated with the common cold is not trivial. The median duration of a cold episode is 7.4 days, with 25% of cases continuing for two weeks. The burden of the common cold is even more pronounced in individuals with chronic co-morbidities or clinical risk factors, including those with asthma and chronic obstructive pulmonary disease, the elderly, those with a history of otitis media or sinusitis, and those who are immunocompromised. Asthmatic children experience more cold episodes than non-asthmatic children, which is a common risk factor for acute asthma exacerbations. Future studies should therefore focus on the role of zinc in these populations rather than healthy people, as the results would be more meaningful for them.

Investigators also need to recognise the difficulties that have been encountered, particularly with respect to blinding and bioavailability (with various formulations). More research is needed in this area. Given the potential for zinc lozenges to induce adverse effects, the zinc lozenges preparation which produces minimal adverse effects should be formulated. We also need more trials on syrup and tablet preparations of zinc as well as more preventive trials in order to support or refute the above mentioned beneficial findings with these preparations.

Although laboratory confirmation of infection is desirable, in large community-based trials the costs associated with such investigations limit the extent to which serology can be undertaken. However, unlike trials relying on experimentally induced rhinoviral colds, findings from large community-based trials will address issues relating to the diversity of and generalisability to the common cold.

Feedback

Zinc for the common cold feedback: the Eby 1984 report, flavor and flavor masking issues, and what is next, 23 February 2011

Summary My congratulations are extended to Meenu Singh and Rashmi R Das for a very comprehensive review of the zinc for common cold literature. However, some comments are needed concerning the Eby et al. 1984 report, flavor issues and where we need to go next.

Concerning the original 1984 Eby, Davis and Halcomb article

Our original 1984 article[1] started this line of inquiry by showing that it was possible to shorten common colds by 7 days (P = 0.0005) with 23 mg zinc (zinc gluconate) lozenges when taken each two wakeful hours. These lozenges (unflavored and unsweetened dietary supplement tablets) lasted about 30 minutes in the mouth which provided sufficient time for local absorption into oral tissues. This protocol was developed after observing that 50 mg zinc (zinc gluconate) tablets slowly dissolved in the mouth of a 3-year old child with acute lymphocytic leukemia (T-cell) resulted in her severe common colds disappearing within 2 hours without further treatment and many similar informal observations in the general community. Our report has drawn some criticism, mainly since the taste of the zinc gluconate lozenges was modestly objectionable resulting in more dropouts in the zinc-treated group than in the placebo-treated group. Bias would occur if zinc-treated subjects who received little or no benefit selectively dropped out or failed to return reports. We were concerned about this potential for

FOI 26-3132 - Document 5

bias, and to protect our results we estimated the maximum effect of such bias by assuming that all dropouts and all non-reporters in both zinc and placebo treatment groups received no benefit and responded as if they were on placebo. The effect of zinc in that analysis remained substantial and statistically strong (P = 0.007), and this potential bias was shown to be irrelevant. These modifications to our main results were published in our Discussion section on page 23 right-hand column, but they have been ignored in reviews by others, thus adversely biasing results of several critical reviews. Therefore, I am disappointed that this error was also committed by Singh and Das.

Flavor and flavor-masking issues

Concern about blinding evolved from concern over “bad taste” of zinc lozenges. There is much difference in the taste of zinc lozenges, which should be considered in evaluating bias. Zinc gluconate in tablets with no other soluble ingredients[1] are moderately objectionable tasting, but tolerable, to most people. Zinc gluconate releases 72% of its zinc as ionic zinc at physiologic pH 7.4.[2] Zinc gluconate can be sweetened without bitterness using a fructose tablet base as was done in the Al-Nakib trial at the Great Britain Common Cold Unit.[3] However, mixing zinc gluconate with any other sweetener, especially sucrose and/or dextrose, results in products that age within 30 days to be as bitter as the bitterest substance known, sucrose octaacetate. This unexpected and adverse effect caused researchers to search for alternatives to zinc gluconate and for ways to flavor-mask zinc gluconate. Extreme bitterness likely affected only the results of Weisman et al., although they compensated by using very small amounts of zinc.[4]

Although pure zinc acetate, which releases 100% ionic zinc at physiologic pH 7.4,[2] tastes vastly more vile than pure zinc gluconate, it is easily flavor-masked by a variety of sweeteners in either hard-candy compositions such as the lozenges tested by Prasad et al.[5] or compressed tablets as tested by Prasad et al.[6] and Petrus et al.[7] These zinc acetate lozenges did not contain flavor-masking additives. Zinc acetate tastes and works best when present in a 1:100 ratio with dextrose in very highly compressed tablets which last about 30 minutes when being dissolved in the mouth. One failure occurred in effervescent lozenges containing zinc acetate which were flavor-masked with tartaric acid and sodium bicarbonate, resulting in elimination of ionic zinc.[8],[9]

Citric acid was used to flavor-mask the objectionable taste of zinc gluconate in a corn syrup and sucrose hard candy lozenge.[10] Citric acid has a high affinity for ionic zinc and no zinc ions were available for absorption into oral tissues, resulting in clinical failure.[2] Due to its pleasant taste, this formula is commonly used in commercial zinc lozenges in the United States resulting in no effect against common colds other than nutritional support.

Glycine was used to flavor-mask zinc gluconate in several clinical trials, with two[11],[12] showing efficacy and one[13] showing lack of efficacy. Variability in results may have been caused by glycine since it eliminates about one half (or more) of the ionic zinc from lozenges.[2] NOTE: This formula does not release “over 90% ionic zinc” at physiologic pH 7.4 as portrayed by Sing and Das, but it does release that amount at pH 5.0, a nonsense pH.

Availability of ionic zinc is dependent upon a number of variables including pH, concentration, temperature and compound being tested, and such is a subject of extensive research by solution equilibrium inorganic chemists .[2]

Consequently, to say that effective zinc lozenges have an objectionable taste that can bias clinical trials is not necessarily factual. The worst possible taste and most bias in effective zinc lozenges results from zinc gluconate in a non-fructose carbohydrate hard candy or tablet base, which was found only in the Weisman trial.[4] Zinc gluconate in a non-soluble tablet base is about 1/10th as objectionable,[1] while zinc gluconate in a fructose tablet base,[3] zinc gluconate-glycine[11, 12, 13] and zinc acetate[5,6,7] in a 1:100 dextrose tablet base are each commercially acceptable in taste. However, zinc acetate lozenges are potentially the most effective, use the least zinc, and are the best tasting. Zinc acetate lozenges also had the least potential for bias due to their pleasant taste, although they had an astringent mouth-feel demonstrating release of zinc ions. A perfect placebo would have been astringent in mouth-feel,

FOI 26-3132 - Document 5

perhaps like tannin. Pursuit of a pleasant tasting and effective zinc lozenge has led to both clinical successes and failures, and the large variety of formulations has greatly complicated the analytical picture.

What is next?

As the researcher that originally discovered the effect of ionic zinc from throat lozenges on the duration of common colds, I am very concerned that the statement "zinc is good for colds" released on nearly every U.S. national TV news program, radio station and newspaper (and perhaps also in Europe, Australia and elsewhere) due to loose journalistic interpretation of the Singh and Das report. It is far too broad and too simplistic to be accurate, and it will likely lead to OTC products that will not have the features necessary to shorten colds more than a day, thus 26 years of zinc lozenge research could be lost and perhaps not regained for decades or a century. These features were previously described in my 2009 review.[2] That review showed dose-response linearity of lozenges by their ionic zinc content on the duration of colds. It showed that reductions in the mean (P=0.001) and median (P=0.004) durations of common colds were statistically significant and meaningful.

It also showed that the effects of a compressed dextrose tablet containing 18 mg of zinc (zinc acetate dyhydrate - 60 mg), glycerol monostearate as a tablet lubricant and peppermint oil on silica gel, which happens to be a 2X homeopathic*Zincum aceticum* formula, is expected to shorten common colds by 7 days. These lozenges released the same amount of ionic zinc as did our 23 mg zinc gluconate lozenges tested in 1984.[1] They are 1.9 cm (¾ inch) diameter and 1.27 cm (1/2 inch) thick tablets which are best produced on heavy production machines like a Stokes 328-2 tablet press. Magnesium stearate was not used as a lubricant due to concerns about concentrated magnesium greatly stimulating rhinovirus replication.[14]

These zinc acetate lozenges are pleasant tasting and taste like peppermint candy. If these tablets are compressed to a sufficient hardness, they will dissolve in the mouth in about 30 minutes. Fick's laws of membrane permeability show that the amount of a solute absorbed across a membrane is time and concentration dependent. Thirty minutes is a reasonable time for oral dissolution. Also, the dextrose-based formula does not excessively promote saliva production. I suggest that it is now time to focus on this basic formulation and go forward with research on it. I see no necessity to focus on zinc compounds that do not release 100% of their zinc as ionic zinc, or on products that dissolve too fast for significant oral absorption, or on products that contain too little ionic zinc to be effective. From the time of contact with the oral mucosa aspects of Fick's laws, the use of syrups or orally ingested tablets to treat common colds are not advised if large reductions in their duration are desired.

Perhaps there is also a requirement to consider how ionic zinc works in shortening colds and the work of Merluzzi et al. in showing that ionic zinc, but not bound zinc, inhibits the replication of rhinoviruses should be considered vital,[15] along with the extensive pioneering work of Korant and Butterworth in showing the inhibitor effects of ionic zinc on rhinoviral replication.[16], [17],[18], [19], [20], [21], [22], [23] These articles should give support to the notion that the main effect of ionic zinc in treating common colds is by rhinovirus replication inhibition.

Although I cannot see into the future, I have proposed here what I believe to be the most likely route to successful treatment of common colds, and I hope that new zinc lozenge researchers will follow my advice. If they do follow my advice, they should be able to demonstrate in a coordinated manner the massive benefits of zinc in treating colds in just a few years, rather than in the decades or centuries that I expect from future distortions of the literature if researchers continue to test faulty products and publish those results. Provided with good results, manufactures can follow through with products that will shorten colds by a week in the general public. Then, and only then, can we say that there is a cure for the common cold!

George Eby

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FOL26-3132 - Document 5

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Reply We thank Dr Eby for his comment on the review as well as giving his expert opinion on the future direction of zinc cold research. Addressing the issue of bias that has occurred in their trial (which has also been highlighted in many other reviews), we want to point out that, though the author (Eby et al) have discussed about the bias resulting from increase drop out in their study in the designated paragraph described above, it should be noted that there are many drawbacks in their trial that make the quality score of a trial poor (please refer to the Cochrane handbook on bias before giving good score to a randomized clinical trial and thus qualifying them for inclusion in a review).

Regarding Dr Eby's description of how zinc acts in the treatment of common cold, it is very true that it is the ionic zinc that plays major role and we hope that authors of future trials will take these issues into account before any meaningful conclusion can be drawn from a particular trial (we have also highlighted these facts in the present review). However, this mechanism might not play important role in the preventive act of zinc for the common cold. But, as zinc has been found in clinical trials to have preventive role for the common cold (zinc also has role in prevention of lower respiratory tract

FOI 26-3132 - Document 5

infection/pneumonia, found in a recent Cochrane review), other mechanisms might play role, including enhancement of innate as well as acquired immunity and correction of underlying zinc deficiency. Only future research in this area can answer these questions.

Contributors Meenu Singh and Rashmi R Das

Comment from Ronald Turner, 1 March 2011

Summary The authors of this review state that our paper (Turner RB, Cetnarowski WE. Effect of treatment with zinc gluconate or zinc acetate on experimental and natural colds. Clin Infect Dis 2000;31:1202-1208) was excluded for poor methodology as the study was not randomized. The study was in fact randomized as stated in the methods section of the paper. If there was confusion on this issue, I would have gladly supplied the original study protocol to the authors of this review.

Ronald Turner Submitter agrees with default conflict of interest statement: I certify that I have no affiliations with or involvement in any organization or entity with a financial interest in the subject matter of my feedback.

Reply This paper by Turner et al, though described as a randomized trial, was exclude because of the poor quality score (not because of randomization only, as commented by the author).

Contributors Meenu Singh and Rashmi R Das

Are we really sure zinc is an effective treatment for the common cold?, 3 March 2011

Summary We are concerned regarding a number of aspects of this updated review on the use of Zinc for the treatment and prevention of the common cold[1]. The review concludes that “zinc is beneficial for the common cold in healthy children and adults living in high-income countries.” We are concerned that the threats of potential biases on the review have not been considered carefully enough. Below we include contour enhanced funnel plots[2] of the two primary treatment outcomes of the review – duration of illness and severity of symptoms (see Figure). Figure: Funnel plots for the two primary treatment outcomes in the review (http://dl.dropbox.com/u/21306757/funnel_plot_zinc.jpg)

Although these plots contain relatively small numbers of studies (6 and 5 respectively), there is a concerning trend in both graphs that the smaller the trials the increasingly larger the effect sizes observed. Additionally, the most precise studies (towards the top of the plots) have effect sizes which are either 0 or very close to it. One possible explanation, and we acknowledge this is not the only explanation, for such “small study effects” trends is publication bias, which would imply that outcomes from trials with less beneficial/harmful effects have been suppressed. This could be due to the suppression of whole trials which have never been published, or, it could be due to suppression of particular outcomes from certain trials (outcome reporting bias)[3] or a combination of the two. Outcome reporting bias is perhaps a particular concern because we infer from the review that 13 treatment trials (including 1 trial excluded from the statistical overview[4]) were identified but only 10 studies reported on each of the primary outcomes, with fewer trials still reporting both outcomes. From this initial position of potential outcome reporting bias, further selection occurred to include these trials into each meta-analysis (6 and 5 respectively), because the review authors believe the results were not reported in a compatible format, although we suggest that it may well be possible to include at least one further study [5] reporting a dichotomous version of the outcome using previously described methods[6, 7]. Some of these studies excluded from the meta-analysis are discussed in the results section, with at least one study having no effect (Douglas 1987[8] for severity of symptoms). Unusually for a review with small study effects, the studies identified as being industry-sponsored are the largest, but are also those closer to the null effect suggesting much smaller effects. The smaller studies indicating greater effects are noted as medical research foundation sponsored, which may support an alternative explanation to the one we propose.

The authors identified heterogeneity between studies and thus used random effects models in the meta-analysis. As this model gives more weight to the smaller, imprecise, studies (than the fixed effect one), in the presence of funnel plot asymmetry, this has the effect of producing larger effect sizes than the fixed effect alternative; both estimates with their 95% confidence intervals are included on the figure

FOI 26-3132 - Document 5

(although both approaches will lead to statistically significant benefits of zinc)[9]. With so few studies, alternative formal approaches to unpicking the heterogeneity, such as meta-regression on dose or dose-duration values would be difficult to undertake.

In the methods section, the authors report making an assessment of publication bias “by examining the funnel plot for asymmetry” and reference a highly cited paper on testing for publication bias[10]. Curiously, there is no further mention or discussion of the results of any assessment for publication bias in the paper. While any formal test on the data is likely to have low power due to the relatively small numbers of studies[6], we believe scrutinising the above funnels is helpful, although the asymmetry is so extreme we initially identified the problem simply by looking at the presented forest plots.

Additionally, we do not understand the author’s statement that “we assessed risk of bias due to selective reporting of outcomes for the study as a whole, rather than for each outcome” For the reasons outlined above, we think some outcome specific consideration would be helpful.

The authors conclude their assessment of “Potential biases in the review process”, that although there was the potential that they may have missed studies referring to the common cold as upper respiratory tract infection, “there are no other obvious sources of potential bias”, we urge the authors to reconsider this statement. In light of the above, and particularly considering the data presented on side effects, we would like to suggest that the conclusions of this review are toned down taking into account the threat of potential reporting biases.

A further issue to have come to light in examining this meta-analysis is that the primary outcome – duration of cold symptoms – is stated to be measured in days. However, the meta-analysis is conducted using the standardised mean difference. If all studies measure the outcome on the same scale (days) it is unclear why the outcome needed standardising and the results would be better presented on the (untransformed) mean difference scale where clinical interpretation is much easier and less strong statistical assumptions are required.

Yours sincerely, Jaime L Peters, Santiago G Moreno, Bob Phillips, Alex J Sutton.

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Submitter agrees with default conflict of interest statement: I certify that I have no affiliations with or involvement in any organization or entity with a financial interest in the subject matter of my feedback.

Reply Thanks for pointing out some important issues related to the review. We also have the same concern regarding the final conclusion (which we have discussed more or less in the review) and that's why no recommendation could presently be made on use of zinc for the common cold. Regarding the publication bias, we agree with the authors (Jaime L Peters, Santiago G Moreno, Bob Phillips, Alex J Sutton) that publication bias could not be ruled out which might also compromise the findings of the

FOI 26-3132 - Document 5

present review. But what's important is that, though publication bias alerts to the problem, they do not provide a solution to it. Moreover, funnel plots have their own limitations. So, what Eby et al described above regarding the future direction is more important, as they provide a solution to the problems in the zinc common cold research. But in the next updated version, we will add the funnel plot. Regarding the application of random and/or fixed model effect in the meta-analysis, they have their inherent problems. Last concern regarding inclusion of MD instead of SMD, we have replied it below [actually, the MD for the duration of cold is -1.48 (around 2 days)].

Contributors Meenu Singh and Rashmi R Das

Comment from Christopher Cates, 7 March 2011

Summary Dear Sir, I have not personally started taking zinc tablets for the common cold, as I do not think the average answer tells me enough to justify a change in practice (too much heterogeneity and a very wide predictive interval [-4.2 to 1.2 days when analyzed as mean difference]. See method in: Riley RD, Higgins JPT, Deeks JJ. Interpretation of random effects meta-analyses. BMJ. 2011;342:d549). Prasad seems to me to be measuring something different to the other studies, as the mean difference of 3 days in these studies is incompatible with all the other study results? It would be easier for readers to understand these results if they were presented as a mean difference in days (rather than SMD). Surely the overall average result is of limited usefulness when there is such diversity between the study results? Yours faithfully, Chris Cates MA FRCGP

Submitter agrees with default conflict of interest statement: I certify that I have no affiliations with or involvement in any organization or entity with a financial interest in the subject matter of my feedback.

Reply The mean difference (in days) for the duration of cold is -1.48 (around 2 days). We agree with the authors regarding the heterogeneity of results, as a result of which no recommendation was possible about zinc for the common cold (already described in conclusion).

Contributors Meenu Singh and Rashmi R Das

Comment from Catherine McIlwain, 14 March 2011

Summary I'd like to request that one line of text (“Zinc inhibits rhinoviral replication”) in the plain language summary be restated in simpler terms. That phrasing resulted in quite a few comments from consumers who don't understand virus replication.

If you are amenable to a change, I would recommend using the word ‘virus’ instead of ‘rhinoviral’ throughout the plain language summary. In addition, would it be acceptable to change ‘rhinoviral replication’ to ‘the spread of the virus’ only in the plain language summary, of course.

Catherine McIlwain

Submitter agrees with default conflict of interest statement: I certify that I have no affiliations with or involvement in any organization or entity with a financial interest in the subject matter of my feedback.

Reply No. replication and spread of virus does not mean necessarily the same thing. But we can say it as follows: zinc inhibits replication of common cold virus.

Contributors Meenu Singh and Rashmi R Das

The zinc for the common cold review by Singh and Das has a number of problems which should be considered when the review is next time updated, 5 September 2011

Summary 1) Search of the studies: Eby and Halcomb (2006) reported a randomized double-blind placebo-controlled trial on zinc for the common cold [1], which is missing from the review. The trial is included in MEDLINE (PMID: 16454145) and Singh and Das should have found it and listed among the trials requiring an assessment. 2) Extraction of data: Singh and Das describe that “The lead review author (MS) entered data directly into Review Manager. An independent coder verified accuracy of data entry”. However, looking at their Analysis 1.1 led me to wonder how can they claim that the findings of the Petrus (1998) trial were non-significant (the 95% CI covering the “no effect”), given

FOI 26-3132 - Document 5

that Petrus (1998 p. 600) wrote that “t-test showed that the mean duration of all symptoms was significantly lower in the zinc group (3.8±0.2 days) than in the placebo group (5.1±0.4 days) (P=0.008)”. In their Analysis 1.1, Singh and Das give the mean duration of colds in the zinc group as 4.4 days, and not as the 3.8 days reported by Petrus (above). The value of 4.4 days is given in the Petrus (1998) Table II as the overall mean duration of colds, i.e. the mean for all zinc and placebo participants combined, but Table II also gives the 3.8 days for the duration of colds in the zinc group. In their paper, Petrus (1998) gives the accuracy of the mean as the SE (standard error), whereas the Review Manager needs the SD (standard deviation). The SE values reported by Petrus (1998) are highly inaccurate (above), yet Singh and Das calculated the corresponding SD estimates from them. To get the accurate Petrus (1998) trial results, I contacted the statistician of the group and got their results: 3.797 (SD 1.630) days in the zinc group and 5.106 (SD 2.955) in the placebo group (Ken Lawson, email 4 Mar 2009). The Petrus (1998) values in Analysis 1.1 should be replaced with these correct and accurate values.

3) The characteristics of included studies table: In the Characteristics of included studies table, Singh and Das write about the Al-Nakib (1987) trial that there was “unclear risk” for the item “incomplete outcome data addressed?”. However, Singh and Das write that “there were no drop-outs or withdrawals”. Lack of drop-outs does not justify their judgment (“unclear risk”). Furthermore, Singh and Das state that in the Al-Nakib trial there is “high risk” for the item “free of selective reporting?”. The statement that there is “high risk” calls for justification, whereas none is given. Furthermore, Singh and Das list six outcomes for the Al-Nakib (1987) trial, yet only one of them is relevant to the review: “the severity of symptoms”. However, I cannot see this outcome in any of their Analysis tables. The outcome section of the included studies table should list only those outcomes that are used in the review. Furthermore, it would help the reader if the included studies table describes in which Analysis the values of the outcomes are presented. Singh and Das state that in the Smith (1987) trial there is “low risk” for the item “incomplete outcome data addressed?”. Smith started with 176 participants and reported the results for only 110 participants, which means that they reported data for only 62% of the initial participants. In their Methods section, Singh and Das write that “we considered that incomplete outcome data had been adequately addressed if 85% or more of the participants were included in the analysis, or if less than 85% were included but adequate steps were taken to ensure or demonstrate that this did not bias the results.” In their included studies table, Singh and Das do not describe why the analysis of 62% of initial participants is acceptable for the Smith (1987) trial although they give the limit of 85% in their Methods. Stating that the Smith (1987) trial has “low risk” with the 38% drop-out rate, and the Al-Nakib (1987) trial has “unclear risk” with no drop-outs (above) is puzzling. Singh and Das use an inclusion criterion “randomized controlled trials”. Weismann (1990) did not report the method of allocation in their trial report. Nevertheless, Singh and Das describe in their included studies table the Weismann (1990) trial: “Methods: randomized trial”. Since their report did not describe the method of allocation, I contacted Kaare Weismann, who wrote to me “It was a consequetive allocated study with the same number of patient in the two groups” (email 2 Jul 2010). Thus, the Weismann (1990) trial was not randomized. Given their inclusion criteria, Singh and Das should exclude the Weismann trial or they should re-write the inclusion criteria so that they also include pseudo-randomized trials which have used, for example, alternative allocation. Furthermore, Singh and Das state that there is “unclear risk” for the item “allocation concealment?” in the Weismann (1990) trial, even though the trial was double blind. The term double-blind means that both the patients and researchers are unaware of the type of treatment until the trial is concluded. Consequently, neither of them can know to which group a patient had been allocated. Thus, double-blind means that there must have been allocation concealment. Otherwise the patients and researchers could not remain blind after randomization. This same error is seen in the description of the Al-Nakib (1987) and Petrus (1998) trials, which also were double-blind trials. As a consequence of this error, Figure 1 gives an impression that several studies were methodologically less satisfactory than they actually were. More details of the trials should be given in the included studies table. For example, in their “implications for practice”, Singh and Das state that “We could not find any trials conducted in low-income countries, so our results cannot be applied to people living in low-income countries.” However, three trials by Kurugol (2006a, 2006b, 2007) were carried out in Turkey, and one trial by Vakili (2009) was carried out in Iran. These are low-income countries. According to World Bank statistics, the GDP per capita in Turkey and Iran is 80% and 90% lower than in Germany, as an example. Thus, the above statement is

FOI 26-3132 - Document 5

false although there are countries which are much poorer than Turkey and Iran. To help the reader to understand the contexts of the trials, it would be useful to describe the country and the settings of the trials. Such information affects the generalization of the findings. Studies in Turkey and Iran cannot be directly extrapolated, for example, to the western countries.

4) The characteristics of excluded studies table: In the Characteristics of excluded studies table, Singh and Das comment on the Eby (1984) trial: “Intention-to-treat analyses were not conducted; analyses were only conducted on a subset of those originally enrolled in the trial.” However, this is also true for the Smith (1987) trial, which included only 62% of the initial participants in the analysis (above). Eby (1984 p. 20-21) described: “Of 146 original volunteers, 120 subjects returned reports. Initially, to use as much of the data as possible, we analyzed the 80 complete reports from 108 subjects who had been ill for 10 days or less at the start of treatment. ... this report is restricted to those 65 subjects who reported being ill for 3 days or less before starting the experiment.” Singh and Das use an inclusion criterion “interventions commenced within three days of participants developing common cold symptoms”. Given such a criterion, Eby's post-randomization restriction to 65 subjects who were ill for 3 days or less before the treatment started is relevant data. In any case, Singh and Das should treat Eby (1984) and Smith (1987) trials consistently, so that both are included or excluded on the basis of the high rate of participants not included in the analysis. Singh and Das further criticise the Eby (1984) trial: “The trial relied on subjective assessment of symptoms by subjects.” However, this applies to essentially all zinc and common cold studies. In evidence-based medicine, the subjective symptoms are most essential outcomes. Subjective symptoms determine whether a patient goes to work or school, stays at home, or visits a physician. Double-blinding prevents systematic bias in the subjective assessment of symptoms, and therefore “subjective” per se cannot cause bias in a double-blind trial. Furthermore, if Singh and Das consider that “subjective assessment” is a basis to exclude the Eby (1984) trial, they should apply the same criterion to the other trials. Singh and Das also comment on the Eby (1984) trial: “Inclusion criteria were not adequately addressed and therefore there may have been potential for selection bias to occur.” In randomized trials, the primary concern is the comparability of trial arms, so that there are no systematic differences that could lead to bias. All randomized trials use inclusion criteria of some kind, but that has nothing to do with the question whether the trial arms are comparable. Inclusion criteria are relevant when we consider the possibility to generalize the results, but not when considering the internal validity of a trial. Finally, Singh and Das conclude their criticism of the Eby (1984) trial: “In addition, no information was provided on how allocation to treatment groups was concealed, the power of the study was not stated and viral studies were not conducted”. First, in most other zinc and common cold studies there is no information about how allocation was concealed (i.e. how blinding was maintained). Second, statistical power is relevant when planning a trial, but not after the trial is concluded, since the confidence interval gives the same information. Third, given that the primary goal in evidence-based medicine is to find out whether a treatment has clinically important effects, viral studies are not relevant. All these issues are missing in most of the zinc and common cold studies. Thus, if Singh and Das consider that these arguments give a sound basis to exclude the Eby trial, they should apply the same criteria also to the other trials. In the Characteristics of excluded studies table, Singh and Das comment on the Turner (2000) trial: “Poor methodological quality. Not a randomised trial”. However, Turner writes “Subjects who met the criteria for randomization to treatment were randomly assigned to 1 of the 4 treatments in accordance with the drug-randomization code” (p. 1202), [for induced colds:] “Subjects were randomized to receive study medication 24 h after challenge if they had a total daily symptom score ≥ 3” (p. 1203), [for natural colds:] “Subjects who presented to the study sites with a common-cold illness of ≤ 1 calendar day’s duration (effectively 36 h), reported ≥ 2 different symptoms, and had a total symptom score of ≥ 2 were randomized to receive 1 of the 3 treatments” (p. 1203).Thus, the statement by Singh and Das is false, unless they have information that disproves the text of the Turner (2000) report. In such a case, they should present their evidence. The Turner (2000) trial was reported as a randomized placebo-controlled double-blind study, and there is no basis to claim that it was of “poor methodological quality”.

5) Different methods of administering zinc should be analyzed separately: The majority of the zinc trials examined zinc lozenges in the western countries. Three trials by Kurugol (2006a, 2006b, 2007) and one trial by Vakili (2009) administered zinc as syrup or tablets; however, all these trials were carried out in low-income countries, Turkey and Iran. Thus, it is possible that the benefit of zinc

FOI-26-3132 - Document 5

supplementation in these trials is caused by biological mechanisms that are different from the mechanisms of the zinc lozenges, which are intended to be dissolved slowly in the mouth. The daily dose of zinc in the Kurugol and Vakili studies varied from 10 to 30 mg per day, whereas the total daily dose of zinc in the zinc lozenge studies varied from 30 to 207 mg per day [2]. Thus, it is possible, or probable, that the benefits of the low dose zinc supplementation found by Kurugol and Vakili are explained by a sub-optimal dietary intake of zinc by children in Turkey and Iran. In contrast, it is possible that high dose zinc is needed in the lozenges to get benefit from them. Although Singh and Das restrict their systematic review to tablets, syrup and lozenges, they should also take a look at the other zinc literature. A few studies have examined the use of nasal administration of zinc to treat colds and found significant benefit [3,4]. Still, patients should not be exposed to intranasal zinc, since there are cases of anosmia caused by such a therapy [5]. Nevertheless, the benefit of local application of zinc to the nose indicates that the effect of zinc lozenges may be caused by local effects in the mouth-throat region, instead of systemic effects such as those caused by the ingestion of tablets and syrup. Therefore it is inappropriate to pool the tablet and syrup trials with the lozenge trials. This is a good example of the apples and oranges problem.

6) The duration of the common cold should not be dichotomized: Singh and Das present three tables which show “number of participants symptomatic after N days of treatment”, N being 3, 5 and 7. Dichotomization of continuous variables has been criticized [6]. Moreover, there is no need to dichotomize the duration of colds when analyzing the zinc lozenge trials. Although several trials did not report the mean and SD for the duration of colds in the trial arms, all of them reported data that makes it possible to calculate the mean and SD for cold duration [2]. The use of continuous outcome for common cold duration would also simplify the review as three redundant tables can be removed.

7) Duration of the common cold should be normalized so that placebo groups have length 100%: There is substantial variation in the duration of colds in the placebo groups of the zinc lozenge trials, from 5.1 days to 9.0 days and 10.8 days [2]. Although part of this variation is evidently caused by random variation, it is also caused by actual variations in the severity of disease in different patient groups and in differences in outcome definitions. Therefore, the relative effect of zinc on the common cold duration should be calculated in percentages, because the relative effect partly adjusts for the variations between patient groups and outcome definitions. For example, if a 6-day cold is shortened by 1 day, it is not equivalent to a 1-day cold being shortened by the same amount although both differences are equal in absolute units. Consequently, it is much more reasonable to calculate the relative effect of zinc, so that a 6-day cold shortened by 2 days and a 1-day cold shortened by 0.33 days both correspond to an equivalent 33% reduction. Calculating the relative effect corresponds to the normalization of all control groups to an episode duration of one unit or 100%. Therefore, in our Cochrane review on vitamin C and the common cold we calculate the relative effect [7]. The use of relative effect in the analysis of common cold duration corresponds to using the risk ratio in the analysis of binary data. In their Analysis 1.1, Singh and Das pool the results by the SMD method, which means normalizing the duration by the SD (i.e. 1 unit in the scale corresponds to the SD of each study). However, using such a scale (SD units) is very difficult for an ordinary reader to understand. In their abstract,, Singh and Das write: “Intake of zinc is associated with a significant reduction in the duration (standardised mean difference (SMD) -0.97)”. However, reporting should always give the unit of the measurement. In the SMD method, the unit is the SD unit. Thus, the above sentence should be re-written more accurately: “zinc shortened the duration of colds by 0.97 SD units”. Such accurate reporting would reveal the main problem of the SMD procedure: what does the SD-unit mean? I pooled the results of three large-dose zinc acetate lozenge trials and I found that “zinc shortened the duration of colds by 42%” [2], which is easy to understand. Most people can form an opinion whether 42% is small or large, but few people can form an opinion whether 0.97 SD units is small or large, or whether it is more or less than 42%. Thus, a relative scale would make the analysis of zinc trials easier to understand for the ordinary readers compared with the SD scale. The Cochrane Handbook comments (9.2.3.2): “The standardized mean difference [SMD] is used as a summary statistic in meta-analysis when the studies all assess the same outcome but measure it in a variety of ways (for example, all studies measure depression but they use different psychometric scales).” Thus, the SMD can be useful, for example, in psychiatry. In the case of common cold duration, the relative effect in percentages is much easier for readers.

FOI 26-3132 - Document 5

8) Subgroup analysis should be carried out: Singh and Das write in the Background section that a "significant correlation between total daily dosages of positively charged zinc species and a reduction in the mean duration of common colds" has been reported. Therefore, the daily dose of zinc should be considered in the statistical analysis. However, in their Result section Singh and Das claim that "subgroup analysis was not possible as there were not enough studies for each variable." This is not correct. As noted above, there is a 6-fold variation in the total zinc dose (30 to 207 mg per day [2]) in the zinc lozenge studies. Given that the results of the zinc lozenge trials diverge so that some found no effect whereas some others found highly significant benefit, the relation between the dose and effect should be analyzed. Dose-response relation is a basic concept in pharmacology. I analyzed 13 zinc lozenge trials and divided them into three subgroups on the basis of the total daily dose of zinc and the type of lozenge [2]. None of five trials with the lowest doses of zinc found benefit of the lozenge, suggesting that they may have been using too low a dose. In the high-dose trials, greater benefit was seen in three trials with zinc acetate, and smaller benefit in five non-acetate trials [2]. Further research should focus, in particular, on high doses of zinc acetate (providing 80-90 mg/day of zinc) [2]. Thus, subgroup analysis is possible and it indicates a path to research that is needed. The syrup and tablet studies with children in the low-income countries should be presented as a separate group, on a separate Analysis table.

9) Pooling the adverse effects of all zinc trials is unsound: Eby has pointed out that the adverse effects of zinc lozenges, such as bad taste, can be explained largely by the differences in the composition of the lozenges [8-10]. In their Discussion, Singh and Das acknowledge this possibility: "the increased incidence of bad taste and nausea ... may have been related to the use of different ligands (gluconate, acetate) rather than to zinc itself." In addition, it is obvious that dissolving a high zinc dose lozenge slowly in the mouth causes different adverse effects compared with ingesting a low zinc dose tablet or syrup straight to the stomach. Nevertheless, Singh and Das combine the adverse effects of the tablet and the zinc lozenge trials together as if they could estimate a "universal adverse effect of zinc" in the dose range of 15 to 192 mg per day. Nevertheless, the lack of "mouth irritation" by zinc syrup in the Kurugol studies (Analysis 2.19) is fully uninformative for a reader who is interested in the possible adverse effects of zinc lozenges. Thus, it would be much more informative to summarize the adverse effects as text, instead of pooling the results of such different trials. In the most recent zinc acetate lozenge trial, there were no significant differences between the zinc and placebo groups in the occurrence of adverse effects although the daily dose was 92 mg (Prasad 2008). Thus, it seems possible to formulate zinc lozenges that have minimal adverse effects. Furthermore, a patient suffering from acute adverse effects such as bad taste can simply stop taking the lozenges, whereas those who don't suffer from such adverse effects could benefit from the lozenges. Although Singh and Das restrict their systematic review to treating the common cold, they should also take a look at other zinc literature for information about zinc safety. For certain patients, zinc has been administered at high doses, 150 mg/day, for therapeutic purposes for months [2,11-12]. On the basis of such long-term studies with high zinc doses, there does not seem to be any basis for assuming that treating the common cold for a week with high doses of zinc (80-90 mg/day) in the form of lozenges might cause unanticipated harm.

10) Credit should be given to earlier work on the same topic: In their Introduction, Singh and Das write "The last review of all available RCTs of zinc for the common cold was published in 1999", which is erroneous. Although it is not reasonable to discuss in detail all the earlier literature on the topic, the main reviews should be cited and briefly commented. Jackson's [13] and Caruso's [14] systematic reviews on zinc and the common cold were published after 1999. In the Discussion section, RevMan proposes a subtitle "Agreements and disagreements with other studies or reviews". Evidently, the same issue can be discussed under some other title. In any case, it would be important for Singh and Das to describe to what extent their review agrees and disagrees with the earlier reviews, such as [13,14]. If the conclusions do not differ from earlier reviews, then a new review does not increase our understanding about the topic. If the conclusions are different, then the reasons for the differences should be briefly discussed.

Harri Hemilä

FOI 26-3132 - Document 5

References [1] Eby GA et al. Ineffectiveness of zinc gluconate nasal spray and zinc orotate lozenges in common-cold treatment: a double-blind, placebo-controlled clinical trial. Altern Ther Health Med 2006;12:34-8.http://www.ncbi.nlm.nih.gov/pubmed/16454145 [2] Hemilä H. Zinc lozenges may shorten the duration of colds: a systematic review. Open Respir Med J 2011;5:51-8.http://dx.doi.org/10.2174/1874306401105010051 [3] Hirt M et al. Zinc nasal gel for the treatment of common cold symptoms: a double-blind, placebo-controlled trial. Ear Nose Throat J 2000;79:778-82.http://www.ncbi.nlm.nih.gov/pubmed/11055098 [4] Mossad SB. Effect of zincum gluconicum nasal gel on the duration and symptom severity of the common cold in otherwise healthy adults. QJM 2003;96:35-43.http://dx.doi.org/10.1093/qjmed/96.1.35 [5] Alexander TH et al. Intranasal zinc and anosmia: the zinc-induced anosmia syndrome. Laryngoscope 2006;116:217-20 and 1721-3.http://dx.doi.org/10.1097/01.mlg.0000191549.17796.13 [6] Royston P et al. Dichotomizing continuous predictors in multiple regression: a bad idea. Stat Med 2006;25:127-41.http://dx.doi.org/10.1002/sim.2331 [7] Hemilä H et al. Vitamin C for preventing and treating the common cold. Cochrane Database Syst Rev 2010:CD000980.http://dx.doi.org/10.1002/14651858.CD000980.pub3 [8] Eby GA. Zinc ion availability - the determinant of efficacy in zinc lozenge treatment of common colds. J Antimicrob Chemother 1997;40:483-93.http://www.ncbi.nlm.nih.gov/pubmed/9372416 [9] Eby GA. Zinc lozenges: cold cure or candy? Solution chemistry determinations. Biosci Rep 2004;24:23-39. http://dx.doi.org/10.1023/B:BIRE.0000037754.71063.41 [10] Eby GA. Zinc lozenges as cure for the common cold – a review and hypothesis. Med Hypotheses 2010;74:482-92. http://dx.doi.org/10.1016/j.mehy.2009.10.017 [11] Hallböök T et al. Serum-zinc and healing of venous leg ulcers. Lancet 1972;2:780-2. http://dx.doi.org/10.1016/S0140-6736(72)92143-5 [12] Simkin PA. Oral zinc sulphate in rheumatoid arthritis. Lancet 1976;2:539-42. http://dx.doi.org/10.1016/S0140-6736(76)91793-1 [13] Jackson JL et al. Zinc and the common cold: a meta-analysis revisited. J Nutr 2000;130(5S Suppl):1512S-5S. http://jn.nutrition.org/cgi/reprint/130/5/1512S [14] Caruso TJ et al. Treatment of naturally acquired common colds with zinc: a structured review. Clin Infect Dis 2007;45:569-74. http://dx.doi.org/10.1086/520031

Submitter agrees with default conflict of interest statement:I certify that I have no affiliations with or involvement in any organization or entity with a financial interest in the subject matter of my feedback.

Reply 1) The Eby and Halcomb (2006) trial has been mentioned under "excluded studies" in the updated review.

2) The Petrus (1998) values in Analysis 1.1 has been replaced with these correct and accurate values in this updated review.

3) About the Al-Nakib (1987) trial, for the item “incomplete outcome data addressed?”, the assessment has been changed into “low risk” in the characterstics of study table. Again, for the Al-Nakib trial, “high risk” for “free of selective reporting?” has been justified in the updated review. Furthermore, regarding the “the severity of symptoms” outcome reported in Al-Nakib (1987) trial, it was not possible to analyse the data, as the same was not avialble and also the trial was conducted almost 26 years back. This might be the reason that, no meta-analysis till date has been able to pool this trial result. Regarding the the Smith (1987) trial, in the updated review it has been changed to “high risk” for the item “incomplete outcome data addressed?”. Regarding the inclusion of Smith (1987) trial in the meta-analysis, please go through the paragraph "dealing with the missing data". Regarding the inclusion of Weismann (1990) trial, it is a randomized trial, and previous meta-analyses (Jackson et al 2000, Hemilä 2011) has handled this as a randomized trial in their analyses. So, there is no point in excluding this trial or lebeling this as a "pseudo-randomized trial". Regarding, contacting the authors, we have already mentioned that we did not try to contact authors of the trials conducted 10 years back. Infact, this trial was conducted almost 20 years back. More details of the Weismann (1990) trial, Al-Nakib (1987) and Petrus (1998) trial have been given in the included studies table in the updated review. The "implications for practice” section has been modified and the sentence “We could not find any trials conducted in low-income countries, so our results cannot be applied to people living in low-income countries” has been removed.

FOI 26-3132 - Document 5

4) Regarding the exclusion of Eby trial (1984), please go through the inclusion criteria and dealing with the missing data section in the updated review. We have remoed the points like “subjective assessment” and "viral studies were not conducted”, as they are not stronger criterion to exclude the Eby (1984) trial. The other points made to exclude the trial are valid and >50% loss to follow up in any trial is not at all acceptable, even if the authors have tried to maintain the integrity of the data. So, we stand on our decision to exclude the Eby trial. Regarding the Turner (2000) trial, it has been included in the updated review.

5) Different methods of administering zinc have been analyzed separately in the updated review.

6) Regarding the duration of the common cold should not be dichotomized, we did not make any change. They are actually not visible, if somone does not really want to visualise them.

7) The duration of the common cold has been normalized so that the placebo group has length 100%. The changes have been made in the updated review.

8) Subgroup analysis has been carried out in the updated review.

9) Though pooling the adverse effects of all zinc trials is unsound, we still reported it as we thought it would be useful and is part of any systematic review. There is no clear cut mechanism postulated and all are assumptions how adverse events occur with zinc lozenges.

10) In the updated version, we have discussed about the earlier reviews by Jackson et al [2000] and Caruso [2007] under the subtitle “Agreements and disagreements with other studies or reviews”. Please go through them.

Contributors Meenu Singh and Rashmi R Das

Zinc for the common cold feedback, 8 April 2014

Summary Were the Hirt and Mossad study that tested intranasal administration excluded from the analysis because of the route of administration? Or were there other reasons as well?

David Riley

I agree with the conflict of interest statement below: I certify that I have no affiliations with or involvement in any organization or entity with a financial interest in the subject matter of my feedback.

Reply We thank David Riley for the feedback on our paper. The Hirt and Mossad study that tested intranasal administration was excluded from the analysis because of the route of administration.

Regards, Dr Rashmi Ranjan and Dr Meenu Singh.

Contributors Meenu Singh and Rashmi R Das

Acknowledgements

We wish to thank Ian IR Marshall, the previous author of this review. We acknowledge all the help and infrastructure provided by the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh and All India Institute of Medical Sciences (AIIMS), New Delhi. We acknowledge the help provided by Elizabeth Dooley, Managing Editor and Sarah Thorning, Trials Search Co-ordinator of the Cochrane Acute Respiratory Infections Group, for the EMBASE search and obtaining full-text articles of studies. We are very grateful to the following referees who commented on drafts of this review: Durhane Wong-Rieger, Ann Fonfa, Anna Joseph, Craig Mellis, Robert Black, Conor Teljeur, Mark Jones and Paul Little.

Appendices

Appendix 1. Details of June 2012 search update

We searched the Cochrane Central Register of Controlled Trials (CENTRAL) 2012, Issue 6, part of *The Cochrane Library*, www.thecochranelibrary.com (accessed 18 June 2012), which contains the Acute Respiratory Infections Group's Specialised Register, MEDLINE (April 2010 to June week 1, 2012),

EQL 26-3132 - Document 5

EMBASE (1974 to June 2012), CINAHL (1981 to June 2012), Web of Science (1985 to June 2012) and LILACS (1982 to June 2012).

We used the following search strategy to search CENTRAL and MEDLINE. We combined the MEDLINE search with the Cochrane Highly Sensitive Search Strategy for identifying randomised trials in MEDLINE: sensitivity- and precision-maximising version (2008 revision); Ovid format (Lefebvre 2011). We adapted the search strategy to search EMBASE, CINAHL, Web of Science and LILACS (see search strategies below).

MEDLINE (OVID)

1 Common Cold/ (3280) 2 common cold*.tw. (2476) 3 Rhinovirus/ (2615) 4 rhinovir*.tw. (3057) 5 Rhinitis/ (7817) 6 rhinit*.tw. (17002) 7 coryza.tw. (355) 8 (respiratory infection* adj3 upper).tw. (1799) 9 (infection* adj3 upper respiratory).tw. (5524) 10 (urti or uri).tw. (890) 11 or/1-10 (33957) 12 Zinc/ (45397) 13 (zinc or zn).tw. (87052) 14 exp Zinc Compounds/ (6713) 15 Micronutrients/ (2677) 16 micronutrient*.tw. (6384) 17 Trace Elements/ (11474) 18 (trace adj2 (mineral* or element*)).tw. (10816) 19 or/12-18 (114540) 20 11 and 19 (237)

EMBASE.com

24. #20 AND #23 23. #21 OR #22 22. random*:ab,ti OR placebo*:ab,ti OR factorial*:ab,ti OR crossover*:ab,ti OR 'cross over':ab,ti OR 'cross-over':ab,ti OR volunteer*:ab,ti OR assign*:ab,ti OR allocat*:ab,ti OR ((doubl* OR singl*) NEAR/2 (blind* OR mask)):ab,ti 21. 'randomized controlled trial'/exp OR 'single blind procedure'/exp OR 'double blind procedure'/exp OR 'crossover procedure'/exp 20. #13 AND #19 19. #14 OR #15 OR #16 OR #17 18. 'trace element':ab,ti OR 'trace elements':ab,ti OR 'trace mineral':ab,ti OR 'trace minerals':ab,ti 17. micronutrient*:ab,ti 16. 'trace element'/de 15. zinc:ab,ti OR zn:ab,ti 14. 'zinc'/exp 13. #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 12. urti:ab,ti OR uri:ab,ti 11. 'upper respiratory tract infection':ab,ti OR 'upper respiratory tract infections':ab,ti OR 'upper respiratory infection':ab,ti OR 'upper respiratory infections':ab,ti 10. 'upper respiratory tract infection'/de 9. rhinit*:ab,ti 8. 'rhinitis'/de 7. coryza:ab,ti 6. rhinovir*:ab,ti 5. 'rhinovirus infection'/de 4. 'rhinovirus'/exp 3. 'common cold symptom'/exp 2. 'common cold':ab,ti OR 'common colds':ab,ti 1. 'common cold'/exp

CINAHL (EBSCO)

S29 S19 and S28 S28 S20 or S21 or S22 or S23 or S24 or S25 or S26 or S27 S27 (MH "Quantitative Studies") S26 TI placebo* OR AB placebo* S25 (MH "Placebos") S24 TI random* OR AB random* S23 TI ((singl* or doubl* or tripl* or trebl*) W1 (mask* or blind*)) OR AB ((singl* or doubl* or tripl* or trebl*) W1 (mask* or blind*)) S22 TI clinic* trial* OR AB clinic* trial* S21 PT clinical trial S20 (MH "Clinical Trials+") S19 S10 and S18 S18 S11 or S12 or S13 or S14 or S15 or S16 or S17 S17 TI (trace W2 (mineral* or element*)) OR AB (trace W2 (mineral* or element*)) S16 (MH "Trace Elements") S15 TI micronutrient* OR AB micronutrient* S14 (MH "Micronutrients") S13 TI (zinc or zn) OR AB (zinc or zn) S12 (MH "Zinc Compounds+") S11 (MH "Zinc") S10 S1 or S2 or S3 or S4 or S5 or S6 or S7 or S8 or S9 S9 TI (urti or uri) OR AB (urti or uri) S8 TI infection* N3 upper respiratory OR AB infection* N3 upper respiratory S7 TI respiratory infection* N3 upper OR AB respiratory infection* N3 upper S6 TI coryza OR AB coryza S5 TI rhinit* OR AB rhinit* S4 (MH "Rhinitis") S3 TI rhinovir* OR AB rhinovir* S2 TI common cold* OR AB common cold* S1 (MH "Common Cold")

Web of Science (Thomson Reuters)

FOI 26-3132 - Document 5

# 5	74	#4 AND #3 *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*	
# 4	1,038,014	Topic=(random* or placebo* or "doubl$ blind$" or "singl$ blind$") OR Title=(trial) *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*	Topic=(random* or placebo* or "doubl$ blind$" or "singl$ blind$") OR Title=(trial) *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*
# 3	216	#2 AND #1 *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*	#2 AND #1 *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*
# 2	289,115	Topic=(zinc or zn or micronutrient* or "trace element$" or "trace mineral$") *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*	Topic=(zinc or zn or micronutrient* or "trace element$" or "trace mineral$") *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*
# 1	26,719	Topic=("common cold$" or rhinovir* or rhinit* or coryza or ("respiratory infection$" NEAR/3 upper) or (infection* NEAR/3 "upper respiratory")) *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*	Topic=("common cold$" or rhinovir* or rhinit* or coryza or ("respiratory infection$" NEAR/3 upper) or (infection* NEAR/3 "upper respiratory")) *Databases=SCI-EXPANDED, CPCI-S, CCR-EXPANDED, IC Timespan=1985-2012 Lemmatization=On*

LILACS (BIREME)

> Search > (MH:"common cold" OR "Resfriado Común" OR "Resfriado Comum" OR "common cold" OR "common colds" OR coryza OR "Coriza Aguda" OR Catarro OR MH:rhinovirus OR rhinovirus OR MH:rhinitis OR rhinit$ OR rinit$ OR "Nasal Catarrh" OR "Catarro Nasal" OR "upper respiratory infection" OR "upper respiratory infections" OR urti or uri) AND (MH:zinc OR zinc or zn or zinco OR MH:"zinc compounds" OR MH:D01.975$ OR "Compuestos de Zinc" OR "Compostos de Zinco" OR MH:"Trace Elements" OR "trace elements" OR "trace element" OR "trace mineral" OR "trace minerals" OR Oligoelementos OR "Elementos Traza" OR MH:Micronutrients OR micronutrient$ OR Micronutrientes)

Appendix 2. Details of previous search strategy

We searched the Cochrane Central Register of Controlled Trials (CENTRAL) (*The Cochrane Library* 2010, Issue 2) which contains the Acute Respiratory Infections Group's Specialised Register, MEDLINE (1966 to May week 3, 2010) and EMBASE (1974 to June 2010). We combined the MEDLINE search with the Cochrane Highly Sensitive Search Strategy for identifying randomised trials in MEDLINE: sensitivity- and precision-maximising version (2008 revision); Ovid format (Lefebvre 2009). See Appendix 2 for the EMBASE search strategy.

FOI 26-3132 - Document 5

MEDLINE (OVID)

1 Common Cold/ 2 common cold*.tw. 3 Rhinovirus/ 4 rhinovir*.tw. 5 Rhinitis/ 6 rhinit*.tw. 7 coryza.tw. 8 (respiratory infection* adj3 upper).tw. 9 (infection* adj3 upper respiratory).tw. 10 (urti or uri).tw. 11 or/1-10 12 Zinc/ 13 (zinc or zn).tw. 14 Micronutrients/ 15 micronutrient*.tw. 16 Trace Elements/ 17 (trace adj (mineral* or element*)).tw. 18 or/12-17 19 11 and 18

EMBASE.com search strategy

24. #20 AND #23 23. #21 OR #22 22. random*:ab,ti OR placebo*:ab,ti OR factorial*:ab,ti OR crossover*:ab,ti OR 'cross over':ab,ti OR 'cross-over':ab,ti OR volunteer*:ab,ti OR assign*:ab,ti OR allocat*:ab,ti OR ((doubl* OR singl*) NEAR/2 (blind* OR mask)):ab,ti 21. 'randomized controlled trial'/exp OR 'single blind procedure'/exp OR 'double blind procedure'/exp OR 'crossover procedure'/exp 20. #13 AND #19 19. #14 OR #15 OR #16 OR #17 18. 'trace element':ab,ti OR 'trace elements':ab,ti OR 'trace mineral':ab,ti OR 'trace minerals':ab,ti 17. micronutrient*:ab,ti 16. 'trace element'/de 15. zinc:ab,ti OR zn:ab,ti 14. 'zinc'/exp 13. #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 12. urti:ab,ti OR uri:ab,ti 11. 'upper respiratory tract infection':ab,ti OR 'upper respiratory tract infections':ab,ti OR 'upper respiratory infection':ab,ti OR 'upper respiratory infections':ab,ti 10. 'upper respiratory tract infection'/de 9. rhinit*:ab,ti 8. 'rhinitis'/de 7. coryza:ab,ti 6. rhinovir*:ab,ti 5. 'rhinovirus infection'/de 4. 'rhinovirus'/exp 3. 'common cold symptom'/exp 2. 'common cold':ab,ti OR 'common colds':ab,ti 1. 'common cold'/exp

Notes

Withdrawn from publication for reasons stated in the review

Data and analyses

FOI 26-3132 - Document 5

Comparison 1

Zinc versus placebo

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1 Duration of cold symptoms	14	1656	Mean Difference (IV, Random, 95% CI)	-1.03 [-1.72, -0.34]
2 Subgroup analysis (duration of cold symptoms)	14	5996	Mean Difference (IV, Random, 95% CI)	-1.07 [-1.43, -0.71]
2.1 Dose of zinc ≥ 75 mg/day	7	620	Mean Difference (IV, Random, 95% CI)	-1.97 [-3.09, -0.85]
2.2 Dose of zinc < 75 mg/day	5	722	Mean Difference (IV, Random, 95% CI)	0.13 [-0.54, 0.79]
2.3 Zinc gluconate lozenges	6	798	Mean Difference (IV, Random, 95% CI)	-0.81 [-1.86, 0.25]
2.4 Zinc acetate lozenges	6	544	Mean Difference (IV, Random, 95% CI)	-1.21 [-2.69, 0.28]
2.5 Zinc lozenges	12	1342	Mean Difference (IV, Random, 95% CI)	-1.04 [-2.02, -0.05]
2.6 Zinc syrup	2	314	Mean Difference (IV, Random, 95% CI)	-0.65 [-0.92, -0.39]
2.7 Children < 16 years age	3	561	Mean Difference (IV, Random, 95% CI)	-0.62 [-0.82, -0.42]
2.8 Adults	11	1095	Mean Difference (IV, Random, 95% CI)	-1.12 [-2.17, -0.06]
3 Severity of cold symptoms	5	513	Mean Difference (IV, Random, 95% CI)	-1.06 [-2.36, 0.23]
4 Subgroup analysis (severity of cold symptoms)	5	513	Mean Difference (IV, Random, 95% CI)	-1.06 [-2.36, 0.23]
4.1 Zinc lozenges	3	199	Mean Difference (IV, Random, 95% CI)	-1.55 [-3.62, 0.52]
4.2 Zinc syrup	2	314	Mean Difference (IV, Random, 95% CI)	-0.27 [-1.51, 0.97]
5 Incidence of common cold	2	1522	Risk Ratio (M-H, Random, 95% CI)	0.64 [0.47, 0.88]

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FOI 26-3132 - Document 5

Comparison 2

Zinc versus placebo

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1 Number of participants symptomatic after 3 days of treatment	3	340	Odds Ratio (M-H, Random, 95% CI)	0.81 [0.27, 2.42]
2 Number of participants symptomatic after 5 days of treatment	3	340	Odds Ratio (M-H, Random, 95% CI)	0.78 [0.32, 1.95]
3 Number of participants symptomatic after 7 days of treatment	5	476	Odds Ratio (M-H, Random, 95% CI)	0.45 [0.20, 1.00]
4 Time to resolution of cough	4	453	Mean Difference (IV, Random, 95% CI)	-1.73 [-3.49, 0.03]
5 Time to resolution of nasal congestion	5	605	Mean Difference (IV, Random, 95% CI)	-0.70 [-1.39, -0.01]
6 Time to resolution of nasal drainage	5	599	Mean Difference (IV, Random, 95% CI)	-1.01 [-2.01, -0.01]
7 Time to resolution of sore throat	4	430	Mean Difference (IV, Fixed, 95% CI)	-0.46 [-0.82, -0.09]
8 Change in cough symptom score	1	101	Mean Difference (IV, Fixed, 95% CI)	-0.23 [-0.26, -0.20]
9 Change in nasal symptom score	2	314	Mean Difference (IV, Fixed, 95% CI)	-0.20 [-1.34, 0.94]
10 School absence (days)	2	394	Mean Difference (IV, Fixed, 95% CI)	-0.66 [-0.99, -0.33]
11 Antibiotic use	2	394	Odds Ratio (M-H, Fixed, 95% CI)	0.27 [0.16, 0.46]
12 Any adverse event	8	1217	Odds Ratio (M-H, Fixed, 95% CI)	1.58 [1.19, 2.09]
12.1 Lozenge formulation	6	897	Odds Ratio (M-H, Fixed, 95% CI)	2.00 [1.40, 2.86]
12.2 Syrup formulation	2	320	Odds Ratio (M-H, Fixed, 95% CI)	1.03 [0.64, 1.66]
13 Bad taste	12	1483	Odds Ratio (M-H, Fixed, 95% CI)	2.31 [1.71, 3.11]
13.1 Lozenges formulation	10	1163	Odds Ratio (M-H, Fixed, 95% CI)	2.66 [1.91, 3.69]

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What's new

Last assessed as up-to-date: 18 January 2013.

FOI 26-3132 - Document 5

Date	Event	Description
17 September 2016	Amended	This Cochrane Review was withdrawn in April 2015, and this withdrawal notice was updated in September 2016. See Published notes.

History

Protocol first published: Issue 1, 1999 Review first published: Issue 2, 1999

Date	Event	Description
30 April 2015	Amended	This review was withdrawn due to concerns raised via the feedback mechanism regarding the calculation and analysis of data in the review in April 2015. Whilst it is not unusual for reviews to be withdrawn, the editorial group took the view that it would be better to take a cautious approach and explore the source and calculation of data used in the analysis in more detail, rather than keep the review on the Cochrane Database of Systematic Reviews for the time being.
29 July 2014	Feedback has been incorporated	Feedback comment added to the review (number 7)
11 December 2013	Amended	Authors have readdressed their reply to the Feedback comment (number 6).
18 October 2013	Amended	Changes made in response to comments received from the Cochrane Editorial Unit
18 January 2013	New citation required but conclusions have not changed	Conclusion regarding the recommendation of zinc for the common cold remains unchanged.
18 January 2013	New search has been performed	Searches updated. Three new trials were included in this updated review (Turner 2000a;Turner 2000b;Turner 2000c) and two new trials were excluded (Eby 2006;Kartasurya 2012).
23 March 2012	Amended	Typographical errors to feedback comment amended.
9 January 2012	Feedback has been incorporated	Feedback incorporated.
7 September 2011	Amended	Authors' replies to Feedback comments added to review.
8 August	Feedback has been	

Characteristics of studies

Characteristics of included studies [ordered by study ID]

FOI 26-3132 - Document 5

Al-Nakib 1987

Methods	Double-blind, placebo-controlled randomised trial
Participants	Healthy adults 18 to 50 years
Interventions	Therapeutic trial: participants took 1 lozenge 2-hourly for 6 days Intervention group: zinc gluconate lozenges containing 23 mg zinc Placebo group: not stated Prophylactic trial: participants took 1 lozenge/2 waking hours for a total of 12 lozenges/day for 4.5 days. On the second day they were challenged with HRV-2 Intervention group: zinc gluconate lozenges containing 23 mg zinc Placebo group: not stated
Outcomes	Severity of symptoms Mean daily nasal secretions Total tissue counts Viral shedding Biochemical and haematological parameters Trial 1: urinary zinc levels
Notes	Although adults were stated to be healthy, no exclusion criteria were stated

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	High risk	Not described. Participants divided into groups balanced by age and sex
Allocation concealment (selection bias)	Unclear risk	The method of concealment was not described in detail to allow a definite judgement
Blinding (performance bias and detection bias) All outcomes	Unclear risk	Insufficient information to permit judgement of ‘low risk’ or ‘high risk’ of bias
Incomplete outcome data (attrition bias) All outcomes	Low risk	There were no drop-outs or withdrawals
Selective reporting (reporting bias)	High risk	1 or more outcomes of interest in the review were insufficiently reported so that they could not be entered in a meta-analysis
Other bias	Unclear risk	The zinc and placebo lozenges were gifted by RBS Pharma, Milan

FOI 26-3132 - Document 5

Douglas 1987

Methods	Double-blind, placebo-controlled randomised trial
Participants	Participants in the trial were healthy adults who had in the previous year participated in a study of interferon prophylaxis against rhinovirus infection
Interventions	Participants took 6 to 8 lozenges/day at 2nd-hourly intervals for a minimum of 3 days and maximum of 6 days if symptoms persisted. New course commenced after 2 weeks if symptoms persisted but type of treatment may differ. Consequently 33 zinc courses and 30 placebo courses Treatment group: zinc acetate lozenges containing 10 mg zinc Placebo group: lozenges contained sodium acetate
Outcomes	Duration and severity of symptoms (nasal, throat or cough) Viral cultures
Notes	The duration of the common cold was ≤ 2 days before starting treatment for 56 of the 58 participants. 2 zinc and 5 placebo treatment courses were excluded because lozenges had not been used for ≥ 3 days and at the rate of ≥ 4 per day. The SD value was not reported and was calculated from P value. The zinc lozenges contained high amount of tartrate; as a result zinc dissociates from acetate and binds instantly to tartrate

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Used sequentially numbered bottles
Allocation concealment (selection bias)	Low risk	Used blocked randomisation in blocks of 4. The code was broken twice (once in middle of study and then at the end of study)
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants and key study personnel ensured. It was unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All		Plausible effect size (difference in means) amongst missing outcomes was

FOI 26-3132 - Document 5

Farr 1987a

Methods	Double-blind, placebo-controlled trial
Participants	Healthy adults
Interventions	Trial 1: treatment consisted of initial loading dose of 2 lozenges 36 hours following inoculation with HRV-39, and thereafter 1 lozenge every 2 hours for a total of 8 lozenges/day for 5 days Intervention group: zinc gluconate lozenges containing 23 mg zinc Placebo group: lozenges contained 0.00125 mg denatonium benzoate Trial 2: treatment consisted of initial loading dose of 2 lozenges 2 hours following inoculation with HRV-13, and thereafter 1 lozenge every 2 hrs for a total of 8 lozenges/day for 7 days Intervention group: zinc gluconate lozenges containing 23 mg zinc Placebo group: 0.0025 mg denatonium benzoate
Outcomes	Severity and duration of symptoms Tissue counts Laboratory tests Infection rates
Notes	Exclusion criteria were symptoms of any respiratory illness in the week before the study, a history of hay fever, any familiarity with the taste of either denatonium benzoate or zinc, a history of any chronic disease, pregnancy, lactation or an unacceptable contraceptive method in women of child-bearing potential, and known abuse of habit-forming drugs

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Computer randomisation
Allocation concealment (selection bias)	Low risk	Each lozenge was wrapped in cellophane and packaged in an opaque polyethylene bottle bearing the study number, the participants' number, the treatment day and dosing instructions
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants and key study personnel ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias)		

FOI 26-3132 - Document 5

Godfrey 1992

Methods	Double-blind, placebo-controlled randomised trial
Participants	Participants were recruited from among Dartmouth College students and staff who spontaneously presented to the cold clinic at the College Health Service. Age ranged from 18 to 40 years. Inclusion required that the cold had lasted ≤ 2 days. Exclusion criteria were positive bacteriological throat culture, pregnancy, lactation and diabetes mellitus
Interventions	Participants took 1 lozenge every 2 hours for up to 8 hours a day Treatment group: zinc gluconate lozenges containing 23.7 mg zinc. Placebo group: lozenges contained tannic acid, glycine and calcium saccharinate in an orange-flavoured, boiled candy base
Outcomes	Frequency and severity of symptoms over 7 days
Notes	The mean duration of the common cold was 1.3 days at entry. 8 zinc and 6 placebo participants withdrew from the trial. The SD value was not reported and was calculated from t-value. The zinc lozenge contained glycine, which binds zinc tightly and therefore the free zinc ion level probably was much lower than suggested by the total zinc dose

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Random numbers table
Allocation concealment (selection bias)	Low risk	A pharmacist, using a randomisation table provided by the study statistician, packaged containers for individual participants with lozenges according to the production run number and subject identification number
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants and key study personnel ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	Plausible effect size (difference in means) amongst missing outcomes was insufficient to have a clinically relevant impact on observed effect size
Selective		

FOI 26-3132 - Document 5

Kurugol 2006a

Methods	Double-blind, placebo-controlled trial	
Participants	The study was conducted at Ege University Nursery and Primary School including children aged 2 to 10 years. Children with chronic disease, immunodeficiency disorder, asthma and history of hypersensitivity were excluded	
Interventions	Therapeutic trial: children received syrup preparation of zinc twice daily for 10 days Intervention group: zinc syrup consisted of 1.32 g zinc sulphate in 100 cm^3 (15 mg of zinc in a 5 cm^3 spoonful) and glycerin, glucose, sunset yellow, orange essence, nipajin Placebo group: similar to above, but lacking the zinc component	
Outcomes	Duration and severity of cold symptoms	
Notes	Used zinc sulfate syrup. A total of 6 (3%) subjects discontinued, 4 for non-compliance and 2 for adverse effects due to medication	
Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Computer random numbers generator
Allocation concealment (selection bias)	Low risk	A statistical consultant programmed a computer-generated randomisation code and prepared the packages of medication
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	Missing outcome data balanced in numbers across intervention groups, with similar reasons for missing data across groups
Selective reporting (reporting bias)	Low risk	The study protocol is not available but it is clear that the published reports include all expected outcomes, including those that were pre-specified
Other bias	Low risk	Berko Ilac Company, Turkey, supplied the active and placebo medications and digital thermometers. The company did not participate in designing the

FOI 26-3132 - Document 5

Kurugol 2006b

Methods	Double-blind, placebo-controlled trial
Participants	The study was conducted at Ege University Nursery and Primary School including children aged 2 to 10 years. Children with chronic disease, immunodeficiency disorder, asthma and history of hypersensitivity were excluded
Interventions	Prophylactic trial: children received syrup preparation of zinc once daily for 7 months. Intervention group: zinc syrup consisted of 1.32 g zinc sulphate in 100 cm^3 (15 mg of zinc in a 5 cm^3 spoonful) and glycerin, glucose, sunset yellow, orange essence, nipajin Placebo group: similar to above, but lacking the zinc component
Outcomes	Number of colds per study child Cold-related school absence Concomitant antibiotic use
Notes	Used zinc sulfate syrup. A total of 6 (3%) participants discontinued, 4 for non-compliance and 2 for adverse effects due to medication

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Computer random numbers generator
Allocation concealment (selection bias)	Low risk	A statistical consultant programmed a computer-generated randomisation code and prepared the packages of medication
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	Missing outcome data balanced in numbers across intervention groups, with similar reasons for missing data across groups
Selective reporting (reporting bias)	Low risk	The study protocol is not available but it is clear that the published reports include all expected outcomes, including those that were pre-specified
Other bias	Low risk	Berko Ilac Company, Turkey, supplied the active and placebo medications and digital thermometers. The company did not participate in designing the

FOI 26-3132 - Document 5

Kurugol 2007

Methods	Double-blind, placebo-controlled trial
Participants	The study was conducted at Ege University Nursery and Primary School including children aged 1 to 10 years. The children who developed symptoms of common cold within the first 24 to 48 hours were registered in the study. Exclusion criteria were common cold symptoms for > 48 hours, immunodeficiency disorder, chronic disease, recent acute respiratory disease (diagnosed by a physician in the previous 2 weeks), zinc allergy, allergic disease or non-allergic rhinitis, positive culture for group A *Streptococcus* and a positive cell culture for influenza A or B viruses
Interventions	Participants were asked to take 1 spoonful of syrup twice a day for 10 days Treatment group: zinc syrup consisted of 1.32 g of zinc sulfate in 100 ml (15 mg of zinc in 5 ml spoonful) and glycerin, glucose, sunset yellow, orange essence and nipajin as preservative Placebo group: identical to above, but lacking the zinc component
Outcomes	Duration and severity of cold symptoms
Notes	Used zinc sulfate syrup. 9 children (3 in the zinc group and 6 in the placebo group) dropped out during the study period because of using antibiotics, decongestants or cough medicine

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Computer random numbers generator
Allocation concealment (selection bias)	Low risk	A statistical consultant programmed a computer-generated randomisation code and prepared the packages of medication. The packages were identical in appearance except for the randomisation numbers. The packages were randomly distributed by the study nurse
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition		

FOI 26-3132 - Document 5

Macknin 1998

Methods	Double-masked, placebo-controlled trial
Participants	Students were recruited from 2 school districts in Cleveland, Ohio. They were aged 6 to 16 years in grades 1 through to 12. Inclusion required that the cold had lasted for ≤ 24 hours. Participants were excluded if they had an oral temperature greater than 37.7ºC, had previously taken the zinc preparation, were pregnant, had a known immune deficiency, any acute illness other than common cold (e.g. pneumonia, gastroenteritis) or cold symptoms lasting more than 24 hours
Interventions	Students asked to take zinc lozenges, 10 mg, orally dissolved, 5 times a day (in grades 1 to 6) or 6 times a day (in grades 7 to 12) until their cold symptoms had been completely resolved for 6 hours Treatment group: zinc gluconate lozenges containing 10 mg zinc in a 3.75 g hard candy that also contained sucrose, corn syrup, glycine Placebo group: lozenges contained calcium lactate pentahydrate instead of zinc and had similar composition as above
Outcomes	Duration of resolution and severity of symptoms
Notes	The median percentage of prescribed lozenges taken was 82.5% in the zinc group. 2 students (1 in each group) provided false information at entry and were excluded from analysis. Mean and SD were not reported and were estimated from the figure. The zinc lozenge contained glycine, which binds zinc tightly and therefore the free zinc ion level probably was lower than suggested by the total zinc dose

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Computer random number generator
Allocation concealment (selection bias)	Low risk	A computer-generated randomisation code was provided to the pharmacist, who held the code and prepared the packages of medication
Blinding (performance bias and detection bias) All outcomes	High risk	Blinding of key study participants and personnel attempted, but likely that the blinding could have been broken

FOI 26-3132 - Document 5

Mossad 1996

Methods	Double-blind, placebo-controlled trial
Participants	Participants were recruited from among the Cleveland Clinic staff through announcements in internal clinic publications and by word of mouth. They were older than 18 years of age. Inclusion required that the cold had lasted for ≤ 24 hours. Exclusion criteria were pregnancy, immune deficiency or symptoms of the common cold for > 24 hours prior to interview
Interventions	Participants took 1 lozenge 2-hourly for every waking hour Treatment group: zinc gluconate lozenges containing 13.3 mg zinc Placebo group: lozenges contained 5% calcium lactate
Outcomes	Duration and severity of cold symptoms
Notes	Participants were assessed for non-adherence to treatment; reasons for non-adherence were: participants had taken antibiotics, condition diagnosed by physician to be other than the common cold, participants filled in diaries from memory, or insufficient lozenges were taken (i.e. fewer than 4 per day for the first 4 days). One participant in the zinc group withdrew from the study on the first day because she could not tolerate the lozenges. Mean and SD were not reported and were estimated from the figure. The zinc lozenge contained glycine, which binds zinc tightly and therefore the free zinc ion level probably was lower than suggested by the total zinc dose

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Computer random numbers generator
Allocation concealment (selection bias)	Low risk	A statistical consultant prepared a computer-generated randomisation code and the packages of medication
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data		

FOI 26-3132 - Document 5

Petrus 1998

Methods	Double-blind, placebo-controlled trial
Participants	Participants were recruited from the campus of the University of Texas through posted announcements. They were 18 to 54 years of age. Participants were excluded if they had a serious illnesses, organ transplants or disability (including HIV infection)
Interventions	Participants were instructed to use a lozenge every 1.5 hours while awake during day 0, then 1 lozenge every 2 hours while awake on following days while symptoms were present for 14 days or 6 hours after disappearance of last symptoms Treatment group: zinc acetate lozenges containing 9 mg zinc in a 2.7 g dextrose base Placebo group: lozenges contained sucrose octaacetate (0.169 mg)
Outcomes	Duration and severity of symptoms
Notes	One subject was lost to follow-up

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	High risk	Not described
Allocation concealment (selection bias)	Unclear risk	The method of concealment is not described or not described in sufficient detail to allow a definite judgement
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants and key study personnel ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	The effect size among missing outcomes not enough to have a clinically relevant impact on observed effect size
Selective		

FOI 26-3132 - Document 5

Prasad 2000

Methods	Double-blind, placebo-controlled trial
Participants	Participants were students, staff and employees at Wayne State University, Michigan, who were ≥ 18 years. Inclusion required that the cold had lasted for ≤ 24 hours. Exclusion criteria were pregnancy, underlying immunodeficiency, chronic illness, symptoms of common cold for more than 24 hours, or had previously used zinc lozenges to treat common cold
Interventions	Participants were asked to use 1 lozenge every 2 to 3 hours while awake for as long as they had symptoms Treatment group: zinc acetate lozenges containing 12.8 mg zinc Placebo group: lozenges contained 0.25 mg of sucrose octaacetate, 6 mg of peppermint oil, 16 mg silica gel, 3877.75 mg dextrose DC and 100 mg glycerol monostearate
Outcomes	Duration of symptoms Plasma levels of zinc and pro inflammatory cytokines
Notes	Two participants in the placebo group dropped out on day 2

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Used randomisation code
Allocation concealment (selection bias)	Low risk	A research consultant prepared the randomisation code and the packages of medication
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	The effect size among missing outcomes not enough to have a clinically relevant impact on observed effect size
Selective		

FOI 26-3132 - Document 5

Prasad 2008

Methods	Double-blind, placebo-controlled trial
Participants	Participants were students, staff and employees at Wayne State University, Michigan, who were ≥ 18 years. Inclusion required that the cold had lasted for ≤ 24 hours. Exclusion criteria were pregnancy, underlying immunodeficiency, chronic illness, symptoms of common cold for more than 24 hours, or had previously used zinc lozenges to treat common cold
Interventions	Participants were asked to use 1 lozenge every 2 to 3 hours while awake for as long as they had symptoms Treatment group: zinc acetate lozenges containing 13.3 mg zinc in a hard candy that contained 3.8 g of sucrose and corn syrup Placebo group: lozenges contained 0.25 mg of sucrose octaacetate
Outcomes	Duration of symptoms Plasma levels of zinc and pro inflammatory cytokines
Notes	No loss to follow-up

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Used randomisation code
Allocation concealment (selection bias)	Low risk	A research consultant prepared the randomisation code and the packages of medication
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	The effect size among missing outcomes not enough to have a clinically relevant impact on observed effect size
Selective		

FOI 26-3132 - Document 5

Smith 1989

Methods	Double-blind, placebo-controlled trial
Participants	Participants were recruited from students from 3 colleges and from 1 family practice. They were older than 18 years. Participants were excluded if they had a serious acute or chronic medical condition, seasonal allergies, productive cough, required antibiotic therapy or had taken any treatment for symptoms within 8 hours of baseline assessment
Interventions	Participants took a loading dose of 4 lozenges then took 2 every 2 hours for 7 days or 24 hours after disappearance of last symptoms Treatment group: zinc gluconate lozenges containing 11.5 mg zinc Placebo group: lozenges contained sucrose octaacetate
Outcomes	Duration and severity of symptoms
Notes	Sixty-four subjects were excluded because of insufficient dose (< 10 lozenges on any day) or insufficient duration of therapy, and 2 were lost to follow-up. Mean and SD were not reported and were estimated from the figure

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Unclear risk	Insufficient information about the sequence generation process to permit judgement of ‘low risk’ or ‘high risk’
Allocation concealment (selection bias)	Unclear risk	The method of concealment is not described in sufficient detail to allow a definite judgement
Blinding (performance bias and detection bias) All outcomes	Low risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	High risk	The effect size among missing outcomes enough to induce clinically relevant bias in observed effect size
Selective reporting		

FOI 26-3132 - Document 5

Turner 2000a

Methods	Double-blind, placebo-controlled trial
Participants	Participants were recruited at 4 different study sites: IMTCI (Lenexa, KS), GFI Pharmaceutical Services (Evansville, IN), TKL Research (Paramus, NJ) and Research Across America (RAA; Dallas). They were 18 to 65 years of age Inclusion required that the cold had lasted for ≤ 1 calendar day (effectively < 36 hrs). Exclusions not described
Interventions	Participants took the lozenges every 2 to 3 hours (a total of 6 per day) while awake until cold symptoms resolved Treatment group: zinc gluconate lozenges containing 13.3 mg zinc Placebo group: lozenges contained tannic acid, sucrose octaacetate, quinine
Outcomes	Duration and severity of symptoms
Notes	Loss to follow-up not described. Analysis was based on intention-to-treat principle. Mean and SD were not reported and were estimated from the figure. The zinc gluconate lozenge contained glycine, which effectively binds to zinc and therefore the free zinc ion level probably was lower than suggested by the total zinc dose

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Used drug randomisation code
Allocation concealment (selection bias)	Unclear risk	The method of concealment is not described in sufficient detail to allow a definite judgement
Blinding (performance bias and detection bias) All outcomes	High risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	Loss to follow-up not described
Selective		

FOI 26-3132 - Document 5

Turner 2000b

Methods	Double-blind, placebo-controlled trial
Participants	Participants were recruited from 4 different study sites: IMTCI (Lenexa, KS), GFI Pharmaceutical Services (Evansville, IN), TKL Research (Paramus, NJ) and Research Across America (RAA; Dallas). They were 18 to 65 years of age Inclusion required that the cold had lasted for ≤ 1 calendar day (effectively < 36 hrs). Exclusions not described
Interventions	Participants took the lozenges every 2 to 3 hours (a total of 6 per day) while awake until cold symptoms resolved Treatment group: zinc gluconate lozenges containing 11.5 mg zinc Placebo group: lozenges contained tannic acid, sucrose octaacetate, quinine
Outcomes	Duration and severity of symptoms
Notes	Loss to follow-up not described. Analysis was based on intention-to-treat principle. Mean and SD were not reported and were estimated from the figure. The zinc gluconate lozenge contained glycine, which effectively binds to zinc and therefore the free zinc ion level probably was lower than suggested by the total zinc dose

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Used drug randomisation code
Allocation concealment (selection bias)	Unclear risk	The method of concealment is not described in sufficient detail to allow a definite judgement
Blinding (performance bias and detection bias) All outcomes	High risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	Loss to follow-up not described
Selective		

FOI 26-3132 - Document 5

Turner 2000c

Methods	Double-blind, placebo-controlled trial
Participants	Participants were recruited from 4 different study sites: IMTCI (Lenexa, KS), GFI Pharmaceutical Services (Evansville, IN), TKL Research (Paramus, NJ) and Research Across America (RAA; Dallas). They were 18 to 65 years of age Inclusion required that the cold had lasted for ≤ 1 calendar day (effectively < 36 hrs). Exclusions not described
Interventions	Participants took the lozenges every 2 to 3 hours (a total of 6 per day) while awake until cold symptoms resolved Treatment group: zinc gluconate lozenges containing 5 mg zinc Placebo group: lozenges contained tannic acid, sucrose octaacetate, quinine
Outcomes	Duration and severity of symptoms
Notes	Loss to follow-up not described. Analysis was based on intention-to-treat principle. Mean and SD were not reported and were estimated from the figure. The zinc gluconate lozenge contained glycine, which effectively binds to zinc and therefore the free zinc ion level probably was lower than suggested by the total zinc dose

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Used drug randomisation code
Allocation concealment (selection bias)	Unclear risk	The method of concealment is not described in sufficient detail to allow a definite judgement
Blinding (performance bias and detection bias) All outcomes	High risk	Blinding of participants, key study personnel and outcome assessment ensured, and unlikely that the blinding could have been broken
Incomplete outcome data (attrition bias) All outcomes	Low risk	Loss to follow-up not described
Selective		

FOI 26-3132 - Document 5

Vakili 2009

Methods	Double-blind, placebo-controlled trial	
Participants	School-aged Iranian children in the suburb of Mashhad. The age range was 6.5 to 10 years. The participants were free from chronic diseases, such as sickle cell disease or protein-energy malnutrition	
Interventions	Participants took tablet daily for 6 days a week for 5 months Treatment group: zinc sulfate tablets containing 10 mg zinc Placebo group: not defined	
Outcomes	Occurrence and duration of common cold	
Notes	Used zinc sulfate tablets. No loss to follow-up	
Risk of bias		
Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	High risk	Not described
Allocation concealment (selection bias)	Unclear risk	The method of concealment is not described in sufficient detail to allow a definite judgement
Blinding (performance bias and detection bias) All outcomes	Unclear risk	Insufficient information to permit judgement of ‘low risk’ or ‘high risk’
Incomplete outcome data (attrition bias) All outcomes	Low risk	No loss to follow-up
Selective reporting (reporting bias)	Low risk	The study protocol is not available but it is clear that the published reports include all expected outcomes, including those that were pre-specified
Other bias	Low risk	This study was supported by grant of vice president for research, Mashhad University of Medical Sciences

FOI 26-3132 - Document 5

Weismann 1990

Methods	Double-blind, placebo-controlled randomised trial
Participants	6 general practitioners residing in the suburban area of Copenhagen conducted the study. Participants were aged 18 to 65 years. Excluded were pregnant and lactating women, diabetics
Interventions	Participants took 1 lozenge at 1 to 1.5-hourly intervals Treatment group: zinc gluconate lozenges (in maltitol syrup) containing 4.5 mg zinc Placebo group: lozenges contained maltitol syrup with natural flavour
Outcomes	Overall assessment of clinical condition assessed by participants using a visual analogue scale
Notes	14 participants were excluded because of a lack of records and 1 because of their young age. Mean and SD were not reported and were estimated from the figure. Consecutive allocation method was used in the study

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	High risk	Not described
Allocation concealment (selection bias)	Unclear risk	The method of concealment is not described in sufficient detail to allow a definite judgement
Blinding (performance bias and detection bias) All outcomes	Unclear risk	Insufficient information to permit judgement of ‘low risk’ or ‘high risk’
Incomplete outcome data (attrition bias) All outcomes	Low risk	The effect size among missing outcomes not enough to have a clinically relevant impact on observed effect size
Selective reporting (reporting bias)	Low risk	The study protocol is not available but it is clear that the published reports include all expected outcomes, including those that were pre-specified
Other bias	Unclear risk	The lozenges were manufactured and supplied by a firm

SD: standard deviation

Characteristics of excluded studies [ordered by study ID]

FOI 26-3132 - Document 5

Study	Reason for exclusion
Eby 1984	The trial was rated of poor methodological quality. A higher incidence of side effects and complaints in the zinc group may have reduced compliance with treatment (no information was provided on whether compliance with treatment was assessed). Intention-to-treat analyses were not conducted; analyses were only conducted on a subset of those originally enrolled in the trial. Inclusion criteria were not adequately addressed and therefore there may have been potential for selection bias to occur. In addition, no information was provided on how allocation to treatment groups was concealed, and the power of the study was not stated.
Eby 2006	Used both zinc gluconate nasal spray and zinc orotate lozenges simultaneously
Kartasurya 2012	Studied upper respiratory tract infection as a whole, used zinc supplementation for 4 months
McElroy 2003	Poor methodological quality. Not a randomised trial
Potter 1993	Poor methodological quality. Not a randomised trial
Veverka 2009	Poor methodological quality. Measured upper respiratory tract infection as a whole (common cold and seasonal flu)

Contributions of authors

Dr Meenu Singh (MS) and Dr Rashmi Ranjan Das (RRD) jointly prepared and edited the review.

Sources of support

Internal sources

- Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India.
- All India Institute of Medical Sciences (AIIMS), New Delhi, India.

External sources

- No sources of support supplied

Declarations of interest

None known.

Notes

September 2016 updated withdrawal notice This Cochrane Review was withdrawn in April 2015, and this withdrawal notice was updated in September 2016.

The review was withdrawn as result of comments submitted via the Cochrane Library by Harri Hemilä in February 2015. Hemilä identified multiple errors in this Cochrane Review and made allegations of plagiarism of text and data from a previously published systematic review (Hemilä H. Zinc Lozenges may shorten the duration of colds: a systematic review. *Open Respiratory Medicine Journal* 2011;5:51-58. dx.doi.org/10.2174/1874306401105010051). The comments referred to the version of this review first published in June 2013 (Singh M, Das RR. Zinc for the common cold. *Cochrane Database of Systematic Reviews* 2013;(6):CD001364. http://onlinelibrary.wiley.com/doi/10.1002/14651858.CD001364.pub4/full).

FOI 26-3132 - Document 5

The Cochrane Acute Respiratory Infections Group, which maintains the review, withdrew the review in April 2015, pending an assessment of the errors reported, and the group referred the allegations of plagiarism to the Editor in Chief. The Editor in Chief notified the authors of the concerns, and followed the Committee for Publication Ethics (COPE) guidelines.

Replication of text was identified in the Cochrane Review. This was limited to copying of short phrases and was acknowledged by the authors. The level of text plagiarism was minor and at a level that would be addressed by a correction. The Editor in Chief carried out further investigation into the alleged plagiarism of data, with the co-operation of the review authors, who provided supplementary information in support of their work. The allegations related to the derivation of means and standard deviations of data from some of the included studies. Although the authors acknowledge and cite the Hemilä 2011 review, the Editor in Chief considered that the authors' explanation regarding some similarities in presented data between the two reviews was not conclusive.

This version of the review will therefore remain withdrawn.

April 2015 withdrawal notice This review was withdrawn due to concerns raised via the feedback mechanism regarding the calculation and analysis of data in the review in April 2015. Whilst it is not unusual for reviews to be withdrawn, the editorial group took the view that it would be better to take a cautious approach and explore the source and calculation of data used in the analysis in more detail, rather than keep the review on the Cochrane Database of Systematic Reviews for the time being.

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Zinc and respiratory tract infections: Perspectives for COVID-19 (Review)

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Received March 23, 2020; Accepted April 13, 2020

DOI: 10.3892/ijmm.2020.4575

Abstract. In view of the emerging COVID-19 pandemic caused by SARS-CoV-2 virus, the search for potential protective and therapeutic antiviral strategies is of particular and urgent interest. Zinc is known to modulate antiviral and antibacterial immunity and regulate inflammatory response. Despite the lack of clinical data, certain indications suggest that modulation of zinc status may be beneficial in COVID-19. *In vitro* experiments demonstrate that Zn^{2+} possesses antiviral activity through inhibition of SARS-CoV RNA polymerase. This effect may underlie therapeutic efficiency of chloroquine known to act as zinc ionophore. Indirect evidence also indicates that Zn^{2+} may decrease the activity of angiotensin-converting enzyme 2 (ACE2), known to be the receptor for SARS-CoV-2. Improved antiviral immunity by zinc may also occur through up-regulation of interferon α production and increasing its antiviral activity. Zinc possesses anti-inflammatory activity by inhibiting NF-κB signaling and modulation of regulatory T-cell functions that may limit the cytokine storm in COVID-19. Improved Zn status may also reduce the risk of bacterial co-infection by improving mucociliary clearance and barrier function of the respiratory epithelium, as well as direct antibacterial effects against *S. pneumoniae*. Zinc status is also tightly associated with risk factors for severe COVID-19 including ageing, immune deficiency, obesity, diabetes, and atherosclerosis, since these are known risk groups for zinc deficiency. Therefore, Zn may possess protective effect as preventive and adjuvant therapy of COVID-19 through reducing inflammation, improvement of mucociliary clearance, prevention of ventilator-induced lung injury, modulation of antiviral and antibacterial immunity. However, further clinical and experimental studies are required.

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Key words: zinc, coronavirus, SARS-CoV-2, pneumonia, immunity

Contents

1. Introduction
2. Zinc and COVID-19
3. Zn and respiratory viruses
4. Pneumonia in adults and the elderly
5. Pediatric respiratory infections
6. Zinc and lung inflammation
7. Zinc and *S. pneumoniae* infection
8. Perspectives and conclusions

1. Introduction

Zinc is an essential metal being involved in a variety of biological processes due to its function as a cofactor, signaling molecule, and structural element. It is involved in the

regulation of carbohydrate and lipid metabolism, as well as the functioning of the reproductive, cardiovascular, and nervous system (1). At the same time, the most critical role of zinc is demonstrated for the immune system. Briefly, zinc regulates proliferation, differentiation, maturation, and functioning of leukocytes and lymphocytes (2). Zinc plays a signaling role involved in the modulation of inflammatory responses (3). It is also a component of nutritional immunity (4). Correspondingly, alteration of zinc status significantly affects immune response resulting in increased susceptibility to inflammatory and infectious diseases including acquired immune deficiency syndrome, measles, malaria, tuberculosis, and pneumonia (5). Earlier data demonstrate that populational Zn status is associated with the prevalence of respiratory tract infections in children and adults (6,7).

In view of the high prevalence of zinc deficiency worldwide (up to 17%), its impact on population health is considered as a significant issue (8). Moreover, certain groups of people, including infants, especially preterm ones, and elderly, are considered to be at high risk of zinc deficiency and its adverse effects (9).

Under zinc deficiency condition, organisms are more susceptible to toxin-producing bacteria or enteroviral pathogens that activate guanylate and adenylate cyclases, stimulating chloride secretion, causing diarrhea and diminishing absorption of nutrients, thus exacerbating an already compromised mineral status. In addition, zinc deficiency may impair the absorption of water and electrolytes, delaying the termination of normally self-limiting gastrointestinal disease episodes (10). During chronic deficiency, the production of pro-inflammatory cytokines increases, influencing the outcome of a large number of inflammatory, metabolic, neurodegenerative and immune diseases (11). Diseases such as rheumatoid arthritis, diabetes (12), atherosclerosis and obesity (13), impaired cognitive function (14), as well as age-related macular degeneration (AMD) may be due to zinc deficiency, worsening chronic inflammation and triggering oxidative stress.

Coronaviridae were considered as the etiological agent in 6-29% of respiratory infections (15,16), although the severity of the disease varies significantly on the particular virus and its virulence (17). The viruses from the *Coronaviridae* family are zoonotic viruses that can be transmitted from animals to humans. The bat is considered the reservoir for these viruses, but other intermediate animals can also transmit the virus to humans (18). COVID-19 is a coronavirus disease caused by the novel 2019-nCoV virus (now called SARS-CoV-2) that appeared for the first time in Wuhan, China at the end of 2019 (19). Despite a close relation other two highly pathogenic coronaviruses, MERS-CoV and SARS-CoV (20), SARS-CoV-2 expanded to the majority of countries (21). On 11 March 2020, WHO characterized COVID-19 as a pandemic (22). Currently, the prevalence of COVID-19 exceeds 1,521,200 cases resulting in 92,700 deaths worldwide (23).

COVID-19 predominantly affects the respiratory system resulting in pneumonia and acute respiratory distress syndrome (24), leading to the requirement of mechanical ventilation (25). In turn, advanced age, acute respiratory distress syndrome (ARDS) and mechanical ventilation are known to be associated with higher COVID-19 mortality (26). The risk is also increased by modern life in which individuals are exposed to a multitude of chemicals, even in low doses that in the long-term predispose to chronic diseases and metabolic disturbances (27-31). Preexisting chronic metabolic diseases including diabetes, cardiovascular diseases (32), and obesity (33) are considered as risk factors for increased COVID-19 susceptibility and mortality. It is proposed that the elderly are at higher risk of COVID-19 due to impaired immune function (34).

Due to the clearly demonstrated role of zinc in immunity (2), and impaired zinc status in ageing (35), metabolic diseases including diabetes, obesity, and cardiovascular diseases (13), it is speculated that zinc compounds may be used as an adjunct therapy in COVID-19 treatment (36) for increasing antiviral resistance (37). Of note, zinc was earlier suggested as the potential agent for immune support and prevention of H1N1 influenza ('swine flu') (38).

In view of lack of clinical data on preventive and/or therapeutic efficiency of zinc in COVID-19, as well as primary involvement of the respiratory system, in this review, we will discuss recent clinical data on the role of zinc in protection against bronchopulmonary infections, as well as the existing indications of the direct impact of zinc on nCoV-2019.

2. Zinc and COVID-19

In view of the global COVID-19 pandemic, potential protective effect of zinc is of particular interest. Zinc is considered as the potential supportive treatment in therapy of COVID-19 infection due to its immune modulatory effect, as well as direct antiviral effect (36). However, the existing data will be only mechanistically discussed in this review, as direct data on anti-COVID-19 effects of zinc are absent to date.

Specifically, Zn^{2+} cations especially in combination with Zn ionophore pyrithione were shown to inhibit SARS-coronavirus RNA polymerase (RNA dependent RNA polymerase, RdRp) activity by decreasing its replication (39). These important findings demonstrate that Zn^{2+} may be considered as the particular antiviral agent in COVID-19 treatment. Of note, recent trials have indicated efficiency of chloroquine antiviral activity as a treatment of COVID-19 (40), although the intimate mechanisms of its antiviral activity require further investigation (41). Earlier findings demonstrate that chloroquine is a zinc ionophore increasing Zn^{2+} flux into the cell (42). Moreover, the authors also propose that chloroquine-mediate zinc influx may underlie anticancer activity of the compound (42). Similarly, it was hypothesized that increasing intracellular Zn^{2+} concentration by chloroquine may also mediate its antiviral effect against SARS-CoV-2. In this view zinc supplementation without chloroquine might have similar positive effects without adverse side-effects of chloroquine treatment (43). Hypothetically, such an effect may be also observed using other zinc ionophores like quercetin and epigallocatechin-gallate (44) with substantially lower toxicity, although clinical trials supported by experimental *in vitro* studies are required to support this hypothesis.

Another Zn-related approach to modulation of COVID-19 may include targeting Zn ions in the structure of viral proteins. Particularly, it has been demonstrated that disulfiram-induced Zn^{2+} release from papain-like protease in MERS-CoV and SARS-CoV resulting in protein destabilization (45). In view of

the presence of similar critical Zn-containing sites, Zn-ejector drugs (e.g., disulfiram) may be considered as potential antiviral agents (46) and components of targeted oxidation strategy in anti-SARS-CoV-2 treatment (47).

SARS-CoV-2 similarly to SARS-CoV requires angiotensin-converting enzyme 2 (ACE2) for entry into target cells (48). Therefore, modulation of ACE2 receptor was considered as the potential therapeutic strategy in COVID-19 treatment (49). Speth *et al* (50) demonstrated that zinc exposure (100 μM) was shown to reduce recombinant human ACE-2 activity in rat lungs. Although this concentration is close to physiological values of total zinc, the modulating effect of zinc on SARS-CoV-2-ACE2 interaction seem to be only hypothetical (51).

Although neither coronavirus HCoV 229E (52) nor HCoV-OC43 (53) infection caused a significant reduction in ciliary beat frequency, HCoV 229E induced ciliary dyskinesia resulting in impaired mucociliary clearance. The latter may not only alter viral particle removal, but also predispose to bacterial co-infection as observed for influenza virus (54). In turn, Zn supplementation was shown to improve ciliary length in bronchial epithelium of Zn-deficient rats (55), as well as increase ciliary beat frequency *in vitro* (56). Therefore, zinc may hypothetically ameliorate nCoV-2019-induced dysfunction of mucociliary clearance. Generally, zinc was shown to be essential for respiratory epithelium due to antioxidant and anti-inflammatory activity (57), as well as regulation of tight junction proteins ZO-1 and Claudin-1 (58), thus increasing its barrier functions. In turn, downregulation of tight junction protein complexes e.g., ZO-1 and Claudin-1 and reduction in barrier function aggravates viral and bacterial inflammatory processes (59). In addition, loss of TJ perm selectivity in the airways results in an un-controlled leakage of high molecular weight proteins and water into the airways, which results in the formation of alveolar edema and ARDS (60).

3. Zn and respiratory viruses

Despite limited data on the direct effect of zinc on SARS-CoV-2 and COVID-19, its antiviral effects were demonstrated in other viral diseases. Zinc was shown to have a significant impact on viral infections through modulation of viral particle entry, fusion, replication, viral protein translation and further release for a number of viruses including those involved in respiratory system pathology (37,61). Specifically, increasing intracellular Zn levels through application of Zn ionophores such as pyrithione and hinokitiol significantly alters replication of picornavirus, the leading cause of common cold (62). These findings generally correspond to the earlier indications of suppressive effect of zinc on rhinovirus replication originating from the early 1970s (63). In addition, Zn treatment was shown to increase interferon α (IFNα) production by leukocytes (64) and potentiate its antiviral activity in rhinovirus-infected cells (65). As antiviral activity of IFNα is mediated through JAK1/STAT1 downstream signaling and up-regulation of antiviral enzymes [e.g., latent ribonuclease (RNaseL) and protein kinase RNA-activated (PKR)] involved in viral RNA degradation and inhibition of viral RNA translation (66), recent findings allow to propose that these mechanisms may be stimulated by Zn^{2+}.

These findings along with the existing data on the role of zinc in immunity raised interest to the potential use of zinc in prevention and/or treatment of common cold. A systematic review by Singh and Das (67) published in Cochrane database revealed a significant reduction in common cold duration, as well as the incidence rate ratio of developing common cold (IRR=0.64 (95% CI: 0.47-0.88), P=0.006) in response to zinc supplementation. The results of meta-analysis demonstrated that Zn supplementation in the dose >75 mg/day significantly reduced duration of common colds (68), with Zn acetate being the most effective form (69).

Certain studies also revealed the association between Zn status and respiratory syncytial virus (RSV) infection. Particularly, it has been demonstrated that whole blood zinc was significantly lower in children with RSV pneumonia (70). Impaired zinc metabolism in perinatal alcohol exposure is associated with immunosuppression and altered alveolar macrophage activity resulting in increased susceptibility to RSV infection (71). In turn, Zn compounds were shown to inhibit respiratory syncytial virus replication and RSV plaque formation with a more than 1,000-fold reduction at 10 μm Zn preincubation (72).

It is also notable that zinc deficiency was associated with higher mortality and adverse long-term outcome in influenza-MRSA bacterial superinfection (73), also underlining the importance of considering the risk of bacterial coinfection.

Despite the presence of experimental findings on the protective effect of zinc supplementation against respiratory virus infections, clinical and epidemiological data are still to be elaborated and systematized.

4. Pneumonia in adults and the elderly

Zinc is essential for the immune system and elderly people have an increased probability for zinc deficiency (74). Low Zn status was considered as the potential risk factor for pneumonia in elderly. Particularly, subjects with high serum Zn (>70 μg/dl, i.e., approx. 10.8 μmol/l) were characterized by reduced incidence of pneumonia [0.52 (0.36, 0.76), P<0.001], as well as lower disease duration and antibiotic administration as compared to low-Zn (<70 μg/ml) group (75), being also related to all-cause mortality (76). Serum Zn levels were 15% lower in cases of community-acquired pneumonia and advanced age, being also associated with pneumonia severity as evaluated by CURB-65 scores (77). The incidence of severe pneumonia was significantly higher in Irani patients with low Zn status, although the mean duration of fever, tachycardia, and tachypnea only tended to be longer, although not significant (78). Correspondingly, serum Zn levels were found deficient at the onset of acute respiratory failure with the lowest values observed in septic shock patients. However, no association between serum Zn values and day-30 mortality or period of stay in intensive care unit was observed (79).

The results of systematic analysis also confirmed the efficiency of intake of at least 75 mg/day Zn in reduction of pneumonia symptom duration but not severity, with the response being more pronounced in adults than in children (80). At the same time, certain studies failed to reveal any improvement in pneumonia when administered along with standard antibiotic treatment, although the period of supplementation was only 4 days (81).

A detailed study by Boudreault *et al* (82) demonstrated that low plasma Zn predisposes to ventilator-induced injury in intensive care, being related to the role of metallothionein system in lung protection. These data corroborate the results of the experimental study demonstrating aggravation of ventilation-induced lung injury in Zn deficient rats (83).

In Indian patients high plasma zinc levels were found to be associated with reduced mortality from sepsis as well as lower 48-h SOFA scores (84). Moreover, persistent low serum Zn levels were associated with increased risk of recurrent sepsis in critically ill patients (85).

Altogether, the existing data demonstrate an association between zinc status and pneumonia in adults and elderly, as well as its complications including respiratory failure, ventilator-induced injury, and sepsis.

5. Pediatric respiratory infections

Initial reports have postulated nearly exceptional susceptibility of elderly to SARS-CoV-2 infection allowing to propose natural resistance to COVID-19 in children (86). However, detailed analysis of the pediatric COVID-19 cases (87) and the emerging Russian experience indicate that children may be also severely affected by SARS-CoV-2. In view of high incidence of Zn deficiency in infants, the existing data on the association between Zn status and pneumonia in children is also discussed.

High incidence of pneumonia in developing countries has been considered as the consequence of zinc deficiency in the population (7). The incidence of low serum zinc in children with severe pneumonia was 80% (88). Correspondingly, a 2-fold lower level of serum Zn was observed in pediatric acute lower respiratory infection patients (89). Significantly lower serum zinc levels were observed in children with pneumonia complicated by sepsis, mechanical ventilation, and cases of lethality (90). Generally, indications of low zinc status in children with pneumonia provide a rationale for preventive Zn supplementation.

Particularly, Zn supplementation in developing countries reduced pneumonia morbidity by 19% (RR=0.81; 95% CI: 0.73, 0.90), whereas a 15% decrease in pneumonia-specific mortality was not significant (91). A recent systematic review and meta-analysis published in Cochrane database demonstrated that Zn supplementation significantly reduced the incidence and prevalence of pneumonia in children by 13 and 41% (92).

In contrast to the demonstrated preventive effects of Zn supplementation, data on the therapeutic effect of zinc in treatment of childhood pneumonia are conflicting (93). Despite the earlier observed reduction of treatment failure risk (94) and case fatality [RR=0.67 (95% CI: 0.24-0.85)] (95) in children with severe pneumonia, a more recent study demonstrated that Zn supplementation in 2-24 months old children with radiologically verified pneumonia did not result in significant improvement of risk reduction of treatment failure (96). Moreover, Zn supplementation in Zn-deficient children with pneumonia until achievement of normal serum Zn levels did not improve clinical appearance of the disease (97).

A number of studies revealed the potential efficiency of Zn supplementation in prevention of non-specified acute lower respiratory infections including bronchitis, bronchiolitis, pneumonitis. Specifically, supplementation with 10 mg zinc gluconate in Zn-deficient children resulted in a nearly twofold reduction of the number of episodes of acute lower respiratory infections as well as the time to recovery (98). In addition, Zn supplementation (30 mg/day) in Thai children significantly reduced severity of acute lower respiratory tract infections resulting in faster disease cessation and shorter hospital stay (99). A detailed meta-analysis demonstrated that Zn supplementation significantly decreased the incidence of acute lower respiratory infection defined according to specific clinical criteria in children aged <5 years (100).

In parallel, the impact of Zn supplementation in relation to upper respiratory tract infections was also demonstrated. Particularly, the number of upper respiratory tract infections in Colombian children was reduced by 73% in response to supplementation with 5 mg Zn in a 12-month randomized clinical trial (101). Certain studies also revealed protective effect of zinc supplementation against both acute upper and lower respiratory diseases in children (102,103).

6. Zinc and lung inflammation

Inflammation plays the key role in COVID-19 pathogenesis both at local (pneumonia) and systemic (cytokine storm) levels, and the search for adequate anti-inflammatory agents is of particular importance (104).

Although the role of zinc in regulation of inflammatory response was discussed in detail in a number of reviews (2,5), certain aspects of the regulatory role of zinc in pneumonia pathogenesis and lung inflammation are still to be elucidated. However, the existing data clearly demonstrate that Zn ions may possess anti-inflammatory effects in pneumonia thus limiting tissue damage and systemic effects.

Specifically, Zn deficiency in rats resulted in a significant increase in proinflammatory TNFα and VCAM-1 expression and lung tissue remodeling, being partially reversed by Zn supplementation (105). Zn deficiency also resulted in a significant alteration of lung epithelial cell barrier function through up-regulation of TNFα, IFNγ, and FasR signaling and cellular apoptosis *in vitro* (106). Zn deficiency was shown to up-regulate acute phase response-related genes through stimulation of JAK-STAT signaling in lungs under septic conditions (107). Zinc and nitric oxide (NO)-metallothioneine (MT)-Zn pathways were shown to mediate lung injury in response to LPS or hyperoxia (108).

In turn, Zn pretreatment significantly reduced LPS-induced pulmonary endothelial cell damage and increased cell viability *in vitro*, as well as improved respiratory function as assessed by blood oxygen pressure and saturation (109). It has been demonstrated that Zn pretreatment significantly decreases LPS-induced neutrophil recruitment to the lungs thus reducing acute lung injury in mice (110).

It is also notable that zinc deficiency is associated with inflammatory alterations of lung extracellular matrix predisposing to fibrosis (111). This finding is of particular interest in view of the presence of interstitial pulmonary fibrosis in COVID-19 patients (112).

Certain studies revealed protective effect of zinc against lung injury in systemic inflammation including sepsis.

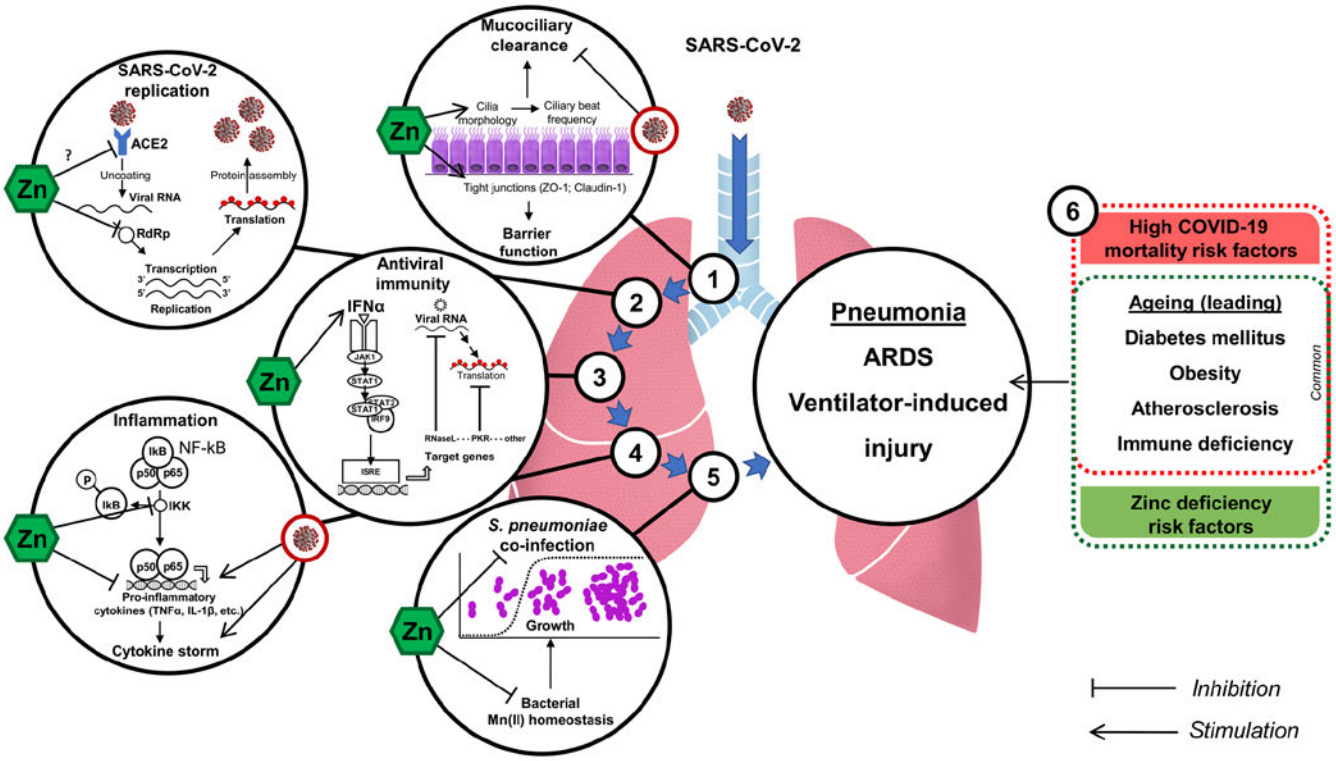


Figure 1. The proposed protective mechanisms of zinc in COVID-19. 1. Zinc significantly improves cilia morphology (54) and increases ciliary beat frequency (55) thus improving mucociliary clearance and removal of bacteria and virus-containing particles. By up-regulating tight junction proteins ZO-1 and claudin-1 (57) and increasing antioxidant activity of respiratory epithelia (56) zinc also increases barrier function of the latter. In turn, coronavirus infection was shown to impair mucociliary clearance (50) predisposing the lung for further viral and bacterial aggression. 2. Zinc may also possess antiviral activity through inhibition of RdRp and blocking further replication of viral RNA as demonstrated for SARS-CoV (38). Indirect evidence also indicates that Zn^{2+} may decrease activity of ACE2 (49), known to be the receptor for SARS-CoV-2 (47). 3. Modulation of antiviral immunity by zinc may also limit SARS-CoV-2 infection at least through up-regulation of IFNα production (63) and increasing its antiviral activity (64). The latter may be mediated through IFNα-induced JAK1/STAT1 signaling and up-regulation of antiviral proteins (RNaseL and PKR) known to degrade viral RNA and inhibit its translation (65). 4. Excessive inflammatory response resulting in overproduction of proiflammatory cytokines and cytokine storm is known to play a significant role in COVID-19 pathogenesis (103). In turn, zinc possesses anti-inflammatory activity through inhibition of IKK activity and subsequent NF-κB signaling resulting in down-regulation of proinflammatory cytokine production (122,124). Modulation of regulatory T-cell functions by Zinc may also limit excessive inflammatory response (125,126) as well as the downregulation of proinflammatory cytokine production (127,123). 5. Given a high risk of bacterial co-infection in viral pneumonia (128), Zn-induced inhibition of *S. pneumoniae* growth through modulation of bacterial Mn(II) homeostasis (137) may also be beneficial. 6. Zinc status is also associated with risk factors for high COVID-19 mortality. Specifically, ageing, immune deficiency, as well as metabolic diseases such as obesity, diabetes, and atherosclerosis, are known to be both risk factors for high disease mortality (31,32) and zinc deficiency (149). In turn, Zn supplementation may have beneficial effect in modulation of at least some of these risk factors. ACE2, angiotensin-converting enzyme 2; IFN, interferon; IKK, IκB kinase; NF-κB, nuclear factor-κB; ARDS, acute respiratory distress syndrome.

Experimental data demonstrate that Zn deficiency increases susceptibility to systemic inflammation and sepsis-induced organ damage including lungs in a murine model of polymicrobial sepsis (113). In a model of polymicrobial sepsis Zn deficiency resulted in increased NF-κB p65 mRNA expression and production in lungs resulting in up-regulation of target genes IL-1β, TNFα, and ICAM-1 (114), whereas Zn supplementation reduced neutrophil infiltration and MPO-mediated oxidative damage (115,116). Modulation of ERK1/2 and NF-κB pathways was shown to be critical for protective effect of zinc in lungs under septic conditions (117).

Correspondingly, patients with sepsis were characterized by low serum Zn levels that may occur due to increased ZIP8 (SLC39A8) mRNA expression. Moreover, serum Zn concentrations inversely correlated with both disease severity and proinflammatory cytokines IL-6, IL-8, and TNFα (118). Reciprocal regulation of ZIP8 and NF-κB expression in response to TNFα or LPS exposure was demonstrated in lung epithelia and alveolar macrophages (119). In addition, ZIP8-deficient mice were characterized by increased airway neutrophil infiltration and elevated CXCL1 and IL-23 production (120).

Zn-mediated respiratory protection was also demonstrated in models of toxic atmospheric pollutant exposure. Particularly, Zn deficiency in agricultural organic dust-exposed animals aggravated neutrophil migration and proinflammatory cytokine (TNFα, IL-6, CXCL1) overproduction, as well as increased IL-23 and CXCL1 expression by macrophages due to NF-κB activation (121). In turn, Zn supplementation in cigarette smoke exposed mice significantly reduced the number of alveolar macrophages in bronchoalveolar lavage (122).

The observed anti-inflammatory effects of Zn in lung tissue seem to be mainly mediated by inhibition of NF-κB signaling through PKA-induced inhibition of Raf-1 and IκB kinase β (IKKβ) (123,124) or A20-dependent inhibition (125). Moreover, Zn-induced modulation of T-cell activity may also play a significant role in limiting inflammatory response (126,127). Lastly, zinc was shown to normalize the overproduction of proinflammatory cytokines induced by zinc deficiency on the epigenetic level (124,128).

7. Zinc and *S. pneumoniae* infection

Although COVID-19 is characterized by viral pneumonia caused by SARS-CoV-2 virus, bacterial co-infection may represent a significant issue due its high incidence in H1N1 influenza-associated pneumonia (129). Specifically, human coronavirus NL63 was associated with increased adherence of *S. pneumoniae* to epithelial cells (130). In turn, *Streptococcus pneumoniae* infection is considered as the most common cause of pneumonia.

Zinc is an essential component of antibacterial immunity (5). Particularly, Zn deficiency was associated with reduced killing activity of phagocytes in pneumococcal infection (131). In turn, Zn supplementation ameliorated the association between nasopharyngeal *S. pneumoniae* carriage and acute lower respiratory infection in children (132). Zn deficiency also predisposed to impaired immune response to Pneumococcal surface protein A, increased nasal *S. pneumoniae* colonization, and severe pneumococcal infection in mice (133) resulting in shorter survival time after infection (134). Correspondingly, patients with better immune response to 23-valent pneumococcal polysaccharide vaccine were characterized by significantly higher serum Zn levels (135). However, no effect (136) or serotype-specific effect (137) of Zn on antibody production in response to polyvalent pneumococcal vaccine was observed. Zn may also exert toxic effect on *S. pneumoniae* reducing its growth through interference with Mn(II) homeostasis and development of cytoplasmic manganese deficiency (138). The latter, in turn, increases bacterial susceptibility to oxygen-dependent killing by neutrophils (139).

A number of studies demonstrated antibacterial effect of zinc oxide nanoparticles (140). Particularly, ZnO was shown to inhibit both growth and biofilm formation by *S. pneumoniae* (141). Similar effect was observed for other bacterial agents involved in etiology of pneumonia, including *K. pneumoniae* (142), methicillin-resistant *S. aureus* (143), and *P. aeruginosa* (144). However, the potential antibacterial application of ZnO-(NPs) may be limited due to their toxicity to human lung cells (145), as well as impairment of phagocytic activity of macrophages in bronchi and lungs (146).

When considering the relationship between *S. pneumoniae* and zinc, one should also note essentiality of Zn ions for bacteria. Specifically, adequate Zn uptake is required for normal bacterial growth and morphology, as well as colonization and virulence (147). Pneumococcal biofilm formation was also shown to be dependent on Zn bioavailability (148).

8. Perspectives and conclusions

The obtained data demonstrate that adequate zinc status of the individual increases immune reactivity. Correspondingly, inadequate zinc supply may predispose to infectious diseases of upper and lower respiratory tract. Although the therapeutic effects of Zn are considered as inconsistent, the existing evidence-based data indicate efficiency of Zn supplementation and improvement of Zn status in prevention of pneumonia and its complications due to anti-inflammatory effect of zinc.

Certain indirect indications of the potential antiviral effect of Zn against nCoV-2019 exist, although their biomedical relevance is yet to be studied. In view of recent data on clinical course of the disease, it appears that adequate Zn status may possess protective effect as adjuvant therapy of COVID-19 through reducing lung inflammation, improvement of mucociliary clearance, prevention of ventilator-induced lung injury, modulation of antibacterial and antiviral immunity especially in elderly (Fig. 1). Further clinical and experimental studies are strongly required to elucidate the potential role of Zn deficiency in COVID-19 susceptibility, as well as effects of Zn supplementation, and the underlying mechanisms.

Acknowledgements

Not applicable.

Funding

The study was partially supported by the Russian Ministry of Science and Higher Education, Project no. 0856-2020-0008. MA was supported by NIH grants nos. NIEHS R0110563, R01ES07331 and NIEHS R01ES020852.

Availability of data and materials

Not applicable.

Authors' contributions

Conceptualization: AVS, LR, MA, JA, AT, AAT; validation, research, resources, data reviewing, and writing: AVS, LR, OPA, MA, VAG, SIA, AAS, DP, DAS, JA, AT, AAT; figure preparation and edition: AAT; review and editing: AVS, LR, MA, JA, AT, AAT. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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FOI 26-3132 - Document 5

Protocol

Efficacy and Safety of High-Dose Ivermectin for Reducing Malaria Transmission (IVERMAL): Protocol for a Double-Blind, Randomized, Placebo-Controlled, Dose-Finding Trial in Western Kenya

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Abstract

Background: Innovative approaches are needed to complement existing tools for malaria elimination. Ivermectin is a broad spectrum antiparasitic endectocide clinically used for onchocerciasis and lymphatic filariasis control at single doses of 150 to 200 mcg/kg. It also shortens the lifespan of mosquitoes that feed on individuals recently treated with ivermectin. However, the effect after a 150 to 200 mcg/kg oral dose is short-lived (6 to 11 days). Modeling suggests higher doses, which prolong the mosquitocidal effects, are needed to make a significant contribution to malaria elimination. Ivermectin has a wide therapeutic index and previous studies have shown doses up to 2000 mcg/kg (ie, 10 times the US Food and Drug Administration approved dose) are well tolerated and safe; the highest dose used for onchocerciasis is a single dose of 800 mcg/kg.

Objective: The aim of this study is to determine the safety, tolerability, and efficacy of ivermectin doses of 0, 300, and 600 mcg/kg/day for 3 days, when provided with a standard 3-day course of the antimalarial dihydroartemisinin-piperaquine (DP), on mosquito survival.

Methods: This is a double-blind, randomized, placebo-controlled, parallel-group, 3-arm, dose-finding trial in adults with uncomplicated malaria. Monte Carlo simulations based on pharmacokinetic modeling were performed to determine the optimum dosing regimens to be tested. Modeling showed that a 3-day regimen of 600 mcg/kg/day achieved similar median (5 to 95 percentiles) maximum drug concentrations (Cmax) of ivermectin to a single of dose of 800 mcg/kg, while increasing the median time above the lethal concentration 50% (LC50, 16 ng/mL) from 1.9 days (1.0 to 5.7) to 6.8 (3.8 to 13.4) days. The 300 mcg/kg/day dose was chosen at 50% of the higher dose to allow evaluation of the dose response. Mosquito survival will be assessed daily up to 28 days in laboratory-reared *Anopheles gambiae s.s.* populations fed on patients' blood taken at days 0, 2 (Cmax), 7 (primary outcome), 10, 14, 21, and 28 after the start of treatment. Safety outcomes include QT-prolongation and mydriasis. The trial will be conducted in 6 health facilities in western Kenya and requires a sample size of 141 participants (47 per arm). Sub-studies include (1) rich pharmacokinetics and (2) direct skin versus membrane feeding assays.

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Results: Recruitment started July 20, 2015. Data collection was completed July 2, 2016. Unblinding and analysis will commence once the database has been completed, cleaned, and locked.

Conclusions: High-dose ivermectin, if found to be safe and well tolerated, might offer a promising new tool for malaria elimination.

(JMIR Res Protoc 2016;5(4):e213) doi: 10.2196/resprot.6617

KEYWORDS

malaria; Plasmodium falciparum; ivermectin; dihydroartemisinin-piperaquine; Anopheles gambiae s.s.; insecticide; clinical trial; pharmacokinetics; Kenya; study protocol

Introduction

Ivermectin is a potential new tool that is being considered in malaria transmission reduction strategies [1]. Ivermectin is a broad spectrum antiparasitic endectocide active against a wide range of internal and external parasites. It was originally introduced as a veterinary drug, predominantly for use in domestic livestock, but since 1987 has been widely used in human medicine [2]. Ivermectin at a dose of 150 or 200 mcg/kg is the first-line treatment for *Onchocerca volvulus* (the cause of river blindness) [3], *Wuchereria bancrofti* (the cause of lymphatic filariasis) [4], and *Strongyloides stercoralis* (roundworm, an intestinal helminth) [5]. To date more than 2.7 billion treatments have been distributed as part of mass drug administration (MDA) [6].

Ivermectin has secondary effects on ectoparasites, such as head lice, mites, bedbugs, and scabies, that feed on recently treated individuals [2,7], and it is also active against *Anopheles spp*. at concentrations present in human blood after standard doses. It reduces the re-blood feeding capacity, female fecundity, hatch rate of their eggs, the survival of progeny larvae, and importantly, it reduces the vector's lifespan [1,8-11]. It may also inhibit parasite sporogony [12]. Ivermectin has a different mode of action from other insecticides, and therefore may be effective against mosquito populations that are resistant to insecticides used on long-lasting insecticidal nets (LLINs) or indoor residual spraying (IRS). Furthermore, it is able to kill exophagic and exophillic vectors that can escape the indoor killing effects of LLINs and IRS [8].

However, several studies have shown that the effects after the standard 150 to 200 mcg/kg doses of ivermectin are generally short-lived. Three *in vivo* studies assessed the long-term effect of ivermectin on mosquito survival by conducting feedings at least 7 days after administration of ivermectin [10,13,14]. A single low dose of 200 mcg/kg showed a 1.33 fold increase in mosquito mortality when fed on blood taken from humans who had received ivermectin 1 day earlier, but there was no longer an effect when mosquitoes were fed on blood taken on day 14 post-treatment [10], while a repeated dose of 200 mcg/kg given on days 0 and 2 showed a modest effect on reduced survival 7 days post-treatment [14], and a dose of 250 mcg/kg in a single human volunteer showed a potent effect for at least 2 weeks post-treatment [13]. Population-based studies of the effect of MDA with ivermectin on malaria transmission or mosquito survival showed that MDA with a single dose of 150 mcg/kg for the control of onchocerciasis in Senegal affected survivorship of *An. gambiae s.s.* for up to 6 days, resulting in an estimated reduction of malaria transmission for at least 11 days as a result of a change in the age-structure of *An. gambiae s.s.* [15-17]. Similarly, in 3 different West African transmission settings, this same dose reduced *An. gambiae* survivorship by 33.9% for 1 week, their parity rates for more than two weeks, and sporozoite rates by more than 77% for 2 weeks [18].

Modeling has also shown that adding 3 days of ivermectin (150 mcg/kg/day) to MDA with dihydroartemisinin-piperaquine (DP) would potentially provide an important boost to the effect of MDAs with artemisinin-based combination therapy (ACT) by allowing transmission to be interrupted faster and in areas with a higher malaria prevalence than MDA with ACTs alone [19]. However, the effects are modest, and higher doses, providing a longer effect are required for ivermectin to boost malaria transmission reduction activities [19].

Ivermectin 400 mcg/kg has been suggested as an improved treatment for head lice [20], and has been found to be safe and well tolerated [21]. No studies in humans have compared the effect of ivermectin doses above 400 mcg/kg on the ability of anopheline vectors to transmit malaria (henceforth referred to as infectivity), or evaluated the effect of any dose of ivermectin higher than 400 mcg/kg on mosquito survivorship.

Ivermectin has an excellent safety profile [1], and experience with higher doses show that it is remarkably well tolerated in humans [22-27], even at doses up to 2000 mcg/kg, 10 times the 200 mcg/kg dose currently approved by the US Food and Drug Administration [24] (Table 1). In invertebrates, ivermectin causes the opening of glutamate-gated chloride channels resulting in flaccid paralysis and death [28]. Glutamate-gated chloride channels do not exist in humans. Other weakly sensitive channels are found in the human central nervous system, but the blood-brain barrier limits drug access to these channels [29].

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Table 1. Studies of safety and tolerability of ivermectin incorporating dosages greater than or equal to 800 mcg/kg.

Reference	Highest single dose	Participants with single dose 800 mcg/kg, n	Total study population, n	Single doses in mcg/kg (n)	Adverse events: increased vs control
Awadzi 1995, 1999 [22,23]	800 mcg/kg	36	100 adult males with onchocerciasis in Ghana	150 (15), 400[a] (25), 600[a] (24), 800[a] (24), 800[b] (12)	No
Guzzo 2002 [24]	2000 mcg/kg	36	68 healthy adults, non-pregnant, in the United States	0 (17), 500[c] (15), 1000[c] (12), 1500 (12), 2000 (12)	No
Kamgno 2004 [25-27]	800 mcg/kg	330	657 adult males with onchocerciasis in Cameroon	150[d] (327), 800[d,e] (330)	Transitory mild visual side effects, without structural abnormalities upon ophthalmological exam

[a]Preceded 3 days earlier by 150 mcg/kg or placebo.

[b]Preceded 13 days earlier by 800 mcg/kg.

[c]Repeated 3 times a week (days 1, 4, and 7).

[d]Repeated 3 times monthly or once yearly.

[e]Preceded 3 or 12 months earlier by 400 mcg/kg.

The only known severe adverse events have been in individuals with *Loa loa*, possibly due to rapid lysis of parasite biomass [30]. Assessment of *Loa loa* is recommended before ivermectin administration in areas endemic for *Loa loa* filariasis [31].

DP and ivermectin have, to the best of our knowledge, never been studied under simultaneous administration. Piperaquine, the long-acting component of DP, is metabolized by, and is an inhibitor of, cytochrome-P450 3A4 (CYP3A4) [32]. There is a potential for an increase of piperaquine plasma concentrations when it is co-administered with other CYP3A4 substrates (due to competition) or CYP3A4 inhibitors [32]. Dihydroartemisinin (DHA), the short-acting component of DP, is not metabolized by cytochrome-P450, but is deactivated via glucuronidation catalyzed by UDP-glucuronosyltransferases, in particular UGT1A9 and UGT2B7 [33]. DHA has been shown to induce CYP3A activity and also up-regulate CYP2C19 and CYP2B6 [33]. DHA is a known inhibitor of CYP1A2 [32].

Ivermectin is primarily metabolized by CYP3A4 [34]. *In vitro* studies using human liver microsomes suggest that ivermectin does not significantly inhibit the metabolizing activities of CYP3A4, CYP2D6, CYP2C9, CYP1A2, and CYP2E1 [34]. When DP and ivermectin are administered together, however, there may be some competition for CYP3A4. The CYP3A4-inhibitory properties of piperaquine may lead to an increased availability of ivermectin. As ivermectin is not a CYP3A4-inhibitor, the potential increase in the availability of piperaquine due to competition is expected to be low.

We will conduct a placebo-controlled dose-finding study to determine the safety, tolerability, and mosquitocidal effect of 3-day courses of ivermectin when given in combination with a standard 3-day course of DP to identify safe and practical regimens to boost the arsenal of available tools to reduce or interrupt malaria transmission. Pharmacokinetic data will be collected to facilitate the construction of a pharmacokinetic/pharmacodynamic (PK/PD) model to guide future study design.

Methods

Design Overview

This is a double-blind, randomized, placebo-controlled, parallel-group, 3-arm, superiority trial to determine the safety, tolerability, and mosquitocidal effect of different doses of ivermectin (ClinicalTrials.gov: NCT02511353). The primary endpoint will be mosquito survival 14 days after a blood feed from a patient who started ivermectin 7 days earlier; 5 days after the last dose of ivermectin with a 3-day regimen administering ivermectin at 0, 24, and 48 hours (days 0, 1 and 2). Because mosquito feeding involves approximately 100 mosquitoes per feed, the study will use a clustered design with the patient as the unit of randomization and the mosquito as the unit of analysis. The study will have a nested rich pharmacokinetic component in the first 36 patients that give additional consent for rich/frequent sampling and a sparse sampling population pharmacokinetic component in the remaining patients. A second nested study will compare the effects of ivermectin when assessed by membrane feeding versus direct skin feeding in all patients who give additional consent for direct skin feeding.

Primary Objective

The primary objective of the study is to determine the safety, tolerability, and efficacy of ivermectin doses of 0, 300 and 600 mcg/kg/day for 3 days, when provided with a standard 3-day course of the antimalarial DP, on mosquito survival.

Secondary Objectives

The secondary objectives of the study are (1) to determine the effect of different doses of ivermectin on oocyst development; (2) to determine the pharmacokinetic profile of the different ivermectin regimens; (3) to determine if ivermectin interacts with the pharmacokinetics of piperaquine; (4) to determine whether the addition of ivermectin to DP affects the clinical and parasitological response to DP treatment; (5) to determine the role of genetic variants of CYP3A4 activity in metabolizing

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ivermectin; and (6) to determine the effect of direct feeding versus membrane feeding on mosquito survival.

Design Considerations

Rationale for Ivermectin Doses of 300 and 600 mcg/kg/day

The goal was to design and evaluate a high-dose ivermectin regimen that could be given daily as adjunct therapy to a 3-day ACT regimen and that builds on the existing safety data available from previous studies. The highest dose of ivermectin used in studies for onchocerciasis is 800 mcg/kg given as a single dose (ie, about 48 mg in an adult male weighing 60 kg). The pharmacokinetic profile of this 800 mcg/kg dose was used to design a 3-day regimen that would achieve a similar maximum drug concentration (Cmax) after the third dose. Since the highest dose of ivermectin used in humans that was tested and found to be well tolerated and safe is 2000 mcg/kg given as a single dose, this provides a large margin of safety allowing for inter-individual variation of pharmacokinetics. The middle group was chosen at 50% of the highest dose to allow for a dose response in terms of tolerance and efficacy.

Using existing literature data [24,35] we developed a pharmacokinetic model for ivermectin in humans. Using the parameter estimates from the model, Monte Carlo simulations were performed for 1000 theoretical participants assuming a 30% variability in parameter estimates (CL/F 11.8 L/h, Vc/F 195.0 L, Q 18.9 L/h, Vp 882 L, and Ka 0.24/h). The simulations showed that the Cmax associated with a single dose of 800 mcg/kg was estimated at 108 ng/ml and the 95% percentile as 164 ng/ml (Figure 1). A regimen of 600 mcg/kg/day for 3 days would give a similar Cmax (111 ng/mL) and corresponding 95% percentile (161 ng/mL) as the single dose 800 mcg/kg regimen (Figure 2 and Table 2). A regimen of 300 mcg/kg/day for 3 days would give approximately half those values. The 3-day regimens were predicted to increase the time that ivermectin concentrations remain above the lethal concentration 50% (LC50) of 16 ng/ml [12] from 46 hours with the 800 mcg/kg single dose to 86 and 162 hours, respectively, with the 300 and 600 mcg/kg/day regimens. The 16 ng/mL threshold was chosen as this was the median of 3 LC50 concentrations reported previously [12,14,15]. The simulated data were in excellent agreement with actual data observed in a dose-finding study by Guzzo et al 2002 [24], which indicated proportional pharmacokinetics at doses ranging from 30 to 120 mg, thus giving confidence in the parameters used in the simulations.

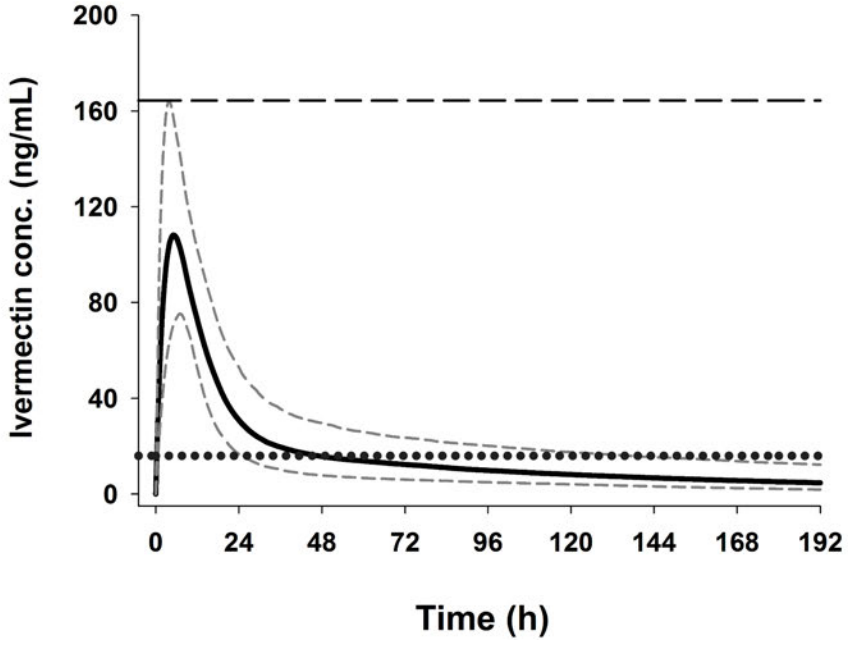
Table 2. Summary of simulated maximum drug concentration and time above lethal concentration 50%.

Ivermectin dosing regimen	Cmax[a] (median 5th-95th percentiles)	Days above LC50[b] (median 5th-95th percentiles)
800 mcg/kg single dose	108.1 (75.3-164.4)	1.9 (1.0-5.7)
600 mcg/kg/day for 3 days	111.0 (83.2-161.2)	6.8 (3.8-13.4)
300 mcg/kg/day for 3 days	55.4 (41.6-80.6)	3.6 (2.8-7.5)

[a]Cmax: maximum drug concentration (ng/mL).

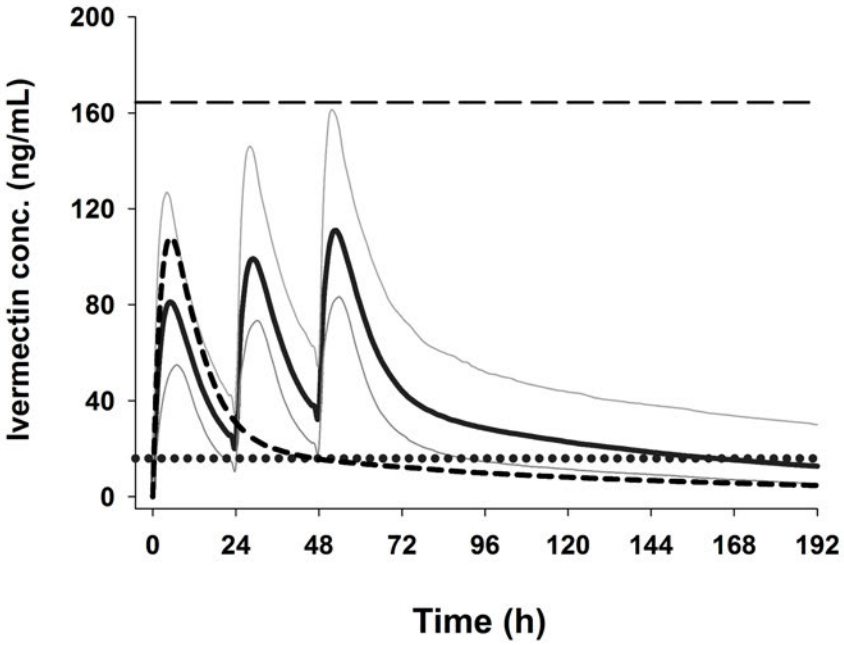
[b]LC50: lethal concentration 50% (16ng/mL).

Figure 1. Simulated plasma concentrations of ivermectin 800 mcg/kg single dose. Monte Carlo simulation of 1000 theoretical subjects of ivermectin concentration with 800 mcg/kg single dose (median: solid line, 5th and 95th percentiles: dashed lines). Cmax is 108.1 ng/mL (CI 75.3-164.4). Time above LC50 (16 ng/mL; dotted line) is 1.9 days (CI 1.0-5.7).



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Figure 2. Simulated plasma concentrations of ivermectin 600 mcg/kg/day 3-day regimen and 800 mcg/kg/day single dose. Monte Carlo simulation of 1000 theoretical subjects of ivermectin concentrations following 600 mcg/kg/day for 3 days (median: solid line, 5th and 95th percentiles: grey lines), achieving similar Cmax concentrations compared to 800 mcg/kg single dose (median: dash curve, 95th percentile of Cmax: dashed horizontal line). The median time above LC50 (16 ng/mL; dotted horizontal line) increases from 1.9 days with 800 mcg/kg single dose to 6.8 days with 600 mcg/kg/day for 3 days.



Parallel Versus Dose-Escalation Design

The proposed study uses a standard parallel design, comparing the 2 intervention arms with the placebo arm. This parallel design, instead of a dose-escalation design (when the lower dose group would be studied first prior to enrolling patients in the higher dose group), was considered appropriate because the Cmax levels and the 95th percentile concentrations in the proposed highest dose group of 600 mcg/kg/day will be equivalent to the Cmax found with single dose 800 mcg/kg, which has been administered to at least 402 patients before as treatment for onchocerciasis or as part of regulatory studies (see Table 1). Furthermore, with 30% variation assumed, the Cmax is estimated to remain well below the Cmax value obtained with 2000 mcg/kg, the highest dose tested and which was well-tolerated in a dose escalation study.

Why Patients with Malaria?

The study will enroll patients with symptomatic uncomplicated malaria, instead of asymptomatic patients with malaria parasites (carriers) or malaria negative individuals who are the predominant target population in MDA campaigns. However, it is unlikely that the mosquitocidal effect of ivermectin will differ much amongst these groups. Preference is given to symptomatic patients based on the rationale that this study is labor intensive, requiring very frequent patient follow-up and blood sampling, and thus requires a major commitment from study participants. Symptomatic patients, aside from similarly requiring antimalarial treatment, are more likely to favor hospital admission and frequent outpatient visits than asymptomatic patients or other volunteers. The frequent follow-up is potentially also more beneficial to the patients with symptomatic malaria than asymptomatic patients.

Justification for Host Genetic Studies

The cytochrome P450s (CYPs) are the major enzymes involved in drug metabolism. To be able to interpret variations in the pharmacokinetic drug profiles of piperaquine and ivermectin, and any drug interactions, we need to determine the genotypes of the genes encoding CYP enzymes (see above).

Direct Skin Feeding Versus Membrane Feeding

The primary endpoint is based on membrane feeding of mosquitoes using blood obtained by venepuncture from patients recently treated with ivermectin. However, a nested sub-study, in all those that give additional consent, will compare mosquito mortality rates between clusters fed using standard membrane feeding versus clusters fed directly (by allowing them to feed on the arm of the study participant). Ivermectin feeding studies with direct feeding on humans [13], and cattle [36], have shown a longer mosquitocidal effect (greater than 2 weeks) in comparison with studies using membrane feeding (less than 7 days) [14].

We hypothesize that direct feeding could result in higher mosquito mortality due to potential differences between venous blood (used in membrane feeding) and blood in subdermal venuoles and arterioles (the main source of blood for mosquitoes during direct skin feeding) due to drug accumulation in subcutaneous fat, dermal, and facial tissue (2- to 3-fold higher concentrations than in venous blood [37]), or increased exposure of the mosquito to ivermectin through other means like perspiration.

There have been no studies conducted directly comparing direct feeding versus membrane feeding on mosquito mortality following ivermectin administration. However, previous studies looking at infectivity (ie, the ability of the vector to develop oocysts and sporozoites after ingesting gametocytes) showed

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FOI 26-3132 - Document 5

significant differences in terms of infectivity in favor of direct feeding (odds ratio 2.39) [38]. Although the mechanisms involved in infectivity studies may differ from studies addressing the killing effect of ivermectin, this recent infectivity study [38] indicates the importance of addressing the potential that the feeding method to expose mosquitoes to ivermectin may be an important effect modifier and that studies using membrane feeding may potentially underestimate the true effect of ivermectin.

Membrane feeding will be used as the primary outcome because direct skin feeding is labor intensive, may be unpleasant to the study participants, and result in higher refusal rates.

Study Setting

The study will be conducted in the Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) in Kisumu, western Kenya, a major tertiary care hospital. Almost 25,000 outpatients are treated for clinical malaria at JOOTRH annually, of which one-third are laboratory-confirmed. Approximately 20% of these patients are 18 to 50 years old. Malaria positive individuals will also be pre-screened at 5 nearby health facilities; those that pass pre-screening and give consent will be brought to JOOTRH for screening and all further study procedures.

Eligibility Criteria

Inclusion and exclusion criteria for the study are shown in Textbox 1.

Textbox 1. Eligibility Criteria

Inclusion Criteria:

- Symptomatic, uncomplicated *P. falciparum* infection
- Positive malaria microscopy or malaria rapid diagnostic test (RDT, pLDH)
- Age 18 to 50 years
- Provide written informed consent
- Agree to be able to travel to clinic on days 1, 2, 7, 10, 14, 21, and 28

Exclusion Criteria:

- Signs or symptoms of severe malaria
- Unable to provide written informed consent
- Women who are pregnant or breast feeding
- Hypersensitivity to ivermectin or DP
- Rate corrected QT interval (QTc) of greater than 460 ms on electrocardiogram (ECG)
- Body mass index (BMI) below 16 or above 32 kg/m2
- Hemoglobin (Hb) concentration below 9 g/dL
- Taken ivermectin in the last month
- Taken DP in the last 12 weeks
- *Loa loa* as assessed by travel history to Angola, Cameroon, Chad, Central African Republic, Congo, DR Congo, Equatorial Guinea, Ethiopia, Gabon, Nigeria and Sudan
- History and/or symptoms indicating chronic illness
- Current use of tuberculosis or anti-retroviral medication
- Previously enrolled in the same study

Trial Medications and Interventions

Participants will be randomized to one of the following 3 arms: (1) the “0 mcg/kg” (placebo) arm consisting of DP plus ivermectin-placebo 600 mcg/kg/day for 3 days, (2) the “300 mcg/kg” arm consisting of DP plus ivermectin 300 mcg/kg/day and ivermectin-placebo 300 mcg/kg/day for 3 days, or (3) the “600 mcg/kg” arm consisting of DP plus ivermectin 600 mcg/kg/day for 3 days. Patients will receive their weight-based doses of DP and ivermectin/placebo. Each dose will be given as directly observed therapy by study staff, after which participants will be monitored for 30 minutes for any vomiting and adverse reactions. If vomiting occurs within 30 minutes, then the participant will be withdrawn from the study, DP will be re-administered, and no further ivermectin will be given.

Dihydroartemisinin-Piperaquine

DP was selected as the drug of choice as it is the most likely candidate to be used in future MDA campaigns because of the longer prophylactic effect against malaria (4 to 6 weeks) compared with 2 to 3 weeks with artemether-lumefantrine (AL). Each participant will receive a weight-based dose of DP 320/40 mg (Eurartesim, Sigma Tau, Italy) as per the product insert (36 to 75 kg: 3 tablets, greater than 75 kg: 4 tablets) once a day for 3 days.

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FOI 26-3132 - Document 5

Ivermectin and Placebo

Ivermectin and/or placebo 6 mg tablets (Iver P, Laboratorio Elea, Argentina) will be administered per bodyweight. The 600 mcg/kg/day arm will receive only ivermectin tablets, the 300 mcg/kg/day arm will receive half the number of ivermectin tablets and an equal number of placebo tablets, and the 0 mcg/kg/day arm will receive only placebo tablets. All participants will receive the same total number of tablets once a day for 3 days based on their bodyweight (45 to 55 kg: 5 tablets, 55 to 65 kg: 6 tablets, 65 to 75 kg: 7 tablets, 75 to 85 kg: 8 tablets, 85 to 95 kg: 9 tablets, 95 to 105 kg: 10 tablets).

Endpoints and Outcome Measures

Primary Efficacy Outcome

The primary efficacy outcome is mosquito survival at 14 days after feeding on blood taken from study participants who started the 3-day ivermectin and DP regimen 7 days earlier (Multimedia Appendix 1).

Secondary Outcomes

Secondary outcomes include (1) mosquito survival at each day, up to day 21 or 28, after each feeding experiments performed at 0, 2 days plus 4h, 7, 10, 14, 21, and 28 days after start of treatment; (2) occurrence of oocysts from day 10 onwards after each feeding as determined by polymerase chain reaction (PCR); (3) malaria clinical and parasitological treatment response by day 28; and (4) plasma concentration profiles of piperaquine and ivermectin as described by standard pharmacokinetic metrics, for example area under the curve measurements from time zero to infinity (AUC0-), time zero to the time of the last measurable concentration (AUC0-tlast), Cmax, and plasma half-life, time to maximum plasma concentration, etc (Multimedia Appendix 1).

Tolerability and Safety Endpoints

Tolerability and safety endpoints are shown in Textbox 2.

Textbox 2. Tolerability and safety endpoints.

- Tolerability
  - Any adverse events assessed in general toxicity questionnaires asked at each study visit
- Safety
  - Primary
  - Mydriasis quantitated by pupillometry [24]
  - Secondary
    - Central nervous system (CNS) effects
    - General toxicity
    - Serious adverse events
    - Hemoglobin concentrations
    - QTc interval (see below “Electrocardiogram Monitoring”)

Participants’ Timeline

Overview Study Phases

The study plan and schedule of assessment is provided in Multimedia Appendix 1. The participant’s timeline will consist of a pre-screening visit (visit 1), consent, screening, and enrolment visit (visit 2), 2 subsequent treatment visits (3 and 4) on days 1 and 2, and 6 follow-up visits for assessment of efficacy parameters (visits 5 to 10). For those enrolled in the pharmacokinetic study additional visits for drug level sample are required as outlined in Multimedia Appendix 2.

Visits 1 and 2: Pre-Screening, Consent, Screening and Enrolment

Patients presenting to the outpatient departments of the study clinics will be pre-screened to determine if they meet readily apparent study eligibility criteria including (1) age 18 to 50 years; (2) uncomplicated malaria; (3) in Kisumu next 4 weeks; (4) hemoglobin (Hb) less than or equal to 9g/dL (if already performed); (5) not pregnant or breast feeding; (6) no known chronic illness; and (7) not previously enrolled in IVERMAL.

Patients passing pre-screening will be approached to obtain consent. For those consenting, study-specific screening procedures will take place, including demographics, full history, past medication use, travel history (*Loa* endemic countries), physical examination, electrocardiogram (ECG), pupillometry, and laboratory tests (to confirm malaria, Hb, and pregnancy). Those fulfilling all enrolment inclusion criteria and not meeting any exclusion criteria will be enrolled into the study, randomized, and treated with the appropriate tablets according to study arm (Textbox 1). Estimated duration is 1.5 to 2.0 hours.

Visits 3 and 4: Treatment Visits

Participants will return to the outpatient clinic on day 1 and 2 for the 2nd and 3rd dose of study drugs. In exceptional cases a participant will be permitted to take the study medication at home or the participant will be visited at home by study staff to administer the medication. A follow-up ECG will be taken just prior to and 4 to 6 hours after the last dose of DP plus ivermectin on day 2.

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FOI 26-3132 - Document 5

Visits 5 to 10: Scheduled Follow-Up Visits

Participants will return to the outpatient clinic for follow-up as specified (see Multimedia Appendix 1). A questionnaire will assess the presence of signs and symptoms, including any adverse effects. A brief clinical examination will be performed and a venous blood sample will be taken for malaria diagnosis, Hb, and drug levels. On visits 5 (day 2 plus 4h) and 6 (day 7), drug levels will also be determined in a finger prick sample. A final follow-up ECG will be taken on the day 28 visit. Participants will be asked to provide telephone numbers so that study staff may make every effort to follow-up participants who have missed scheduled visits (Multimedia Appendix 2, section 8.5.5, page 31).

Unscheduled Visits

At any time, participants displaying signs or symptoms of severe malaria will be admitted to the inpatient ward for further evaluation and treatment free of charge. Blood samples for malaria smears, parasite genetics (filter paper dried blood spots) and Hb will be taken if clinically indicated (e.g. documented fever greater than or equal to 37.5°C axillary, or a history of fever in the last 24 hours).

Sample Size

The study requires a total of 141 participants (47 participants in the 0, 300, and 600 mcg/kg/day groups each). This is powered at 80% to detect a relative increase of 30% (RR 1.300) in the 14-day post-feeding mortality rate (primary outcome) from 24% in the control group (0 mcg/kg ivermectin) to 31.2% in the 300 mcg/kg/day group, and a 25% (RR 1.246) increase from 31.2% with 300 mcg/kg/day to 38.9% in 600 mcg/kg/day recipients, measured from blood taken 7 days after the start of intake of ivermectin and using clusters of 100 anopheline mosquitoes allowing for 10% non-feeders (alpha=.05). The same sample size would give 90% power to detect a 35% (RR 1.348) increase from 24% (0 mcg/kg/day) to 32.4% (300 mcg/kg/day), and 27.7% increase (RR 1.285) from 32.4% (300 mcg/kg/day) to 41.3% (600 mcg/kg/day). The calculations assume an intracluster correlation coefficient (ICC) of .0622 and allow for 6.5% loss-to follow-up of participants by day 7 (ie, 44 of the 47 patients per arm contribute to the primary analysis) [14]. The 10% non-feeding rate is based on current data from the same laboratories at Kenya Medical Research Institute (KEMRI), Kisian, Kenya. The 24% mortality rate estimate by day 14 post-feeding in the control arm is average of observation at KEMRI (18.3%) and in a recent study in Burkina Faso, which showed a 21.2% mortality by day 10 [14], which when extrapolated with 4 additional days predicted a mortality of 29.7% by day 14. The ICC value of .0622 was calculated using data from the recent study in Burkina Faso (Bousema, personal communications) [14].

Assignment of Interventions

Allocation

The study will use stratified randomization (4 strata) by body mass index (BMI: high/low) and sex (male/female) as these are important determinants of the pharmacokinetics of ivermectin [14]. The high/low BMI thresholds are 23 kg/m^2 and 21 kg/m^2, for females and males respectively. Participants will be randomly assigned to 1 of the 3 study arms. The study statistician will computer-generate a randomization sequence using permuted block randomization with fixed block sizes.

Blinding

The study will be double-blinded to participants and study staff. Allocation concealment will be achieved by use of sealed opaque envelopes. All study participants in all 3 arms will receive standard dose DP, and also active (600 mcg/kg/day arm), placebo (0 mcg/kg/day arm), or a combination of active and placebo ivermectin tablets (300 mcg/kg/day arm), such that each arm receives the same number of tablets in each weight strata.

Pharmacokinetic Studies

Overview

The first 36 patients to give additional consent for rich pharmacokinetics (approximately 12 per arm), will be enrolled in a rich pharmacokinetic study using frequent sampling per individual (26 samples per patient, see Multimedia Appendix 2 [Table 2, page 15]) to determine the detailed pharmacokinetic profile of the 2 regimens and assess whether any drug interaction occurs with piperaquine that is of clinical relevance. The remaining patients (approximately 35 per arm) will contribute to a population pharmacokinetic study consisting of sparse pharmacokinetic sampling with a maximum of 13 samples per patient including baseline (1 venous sample), 6 scheduled visits as part of the main trial (6 venous and 2 finger prick samples), and 2 extra visits for population pharmacokinetic sampling (2 venous and 2 finger prick samples).

The rich and population pharmacokinetic studies combined will allow us to determine the main sources and correlates of variability in drug concentrations (for both ivermectin and piperaquine), including demographic, pathophysiological, such as BMI and gender, and other factors that might alter dose-concentration relationships. As this is a placebo controlled trial, the sampling methodology for the 47 patients in the ivermectin-placebo arm will be identical to that used for the 300 and 600 mcg/kg arms. The patients in the placebo-ivermectin arm will allow us to determine the piperaquine kinetic profile in the absence of ivermectin.

Finger prick blood draws will be performed at a maximum of 4 time points in addition to the venous blood draws. The aim is to compare the capillary and venous drug concentration levels as it has been hypothesized that these might differ for ivermectin, similar to other drugs including piperaquine. A difference between capillary and venous drug concentrations could help further explain any observed difference in mosquito mortality between membrane and direct skin feeding (see also above “Direct Skin Feeding versus Membrane Feeding”).

Standard Pharmacokinetic Study (Rich Sampling)

All of the rich pharmacokinetic participants (approximately 12 per arm) will have venous blood sampled (4 ml whole blood to obtain 2 ml plasma, or 5.2 mL of whole blood if coinciding with a scheduled follow-up visit for the main trial) at baseline and each of 21 follow-up time points listed in Multimedia Appendix

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FOI 26-3132 - Document 5

2 (Table 2, page 15). In addition, 4 finger pricks (0.5 mL whole blood) will be taken at days 2 plus 4 h, 3, 4 and 7. The total blood volume to be drawn from these patients is 98.4 mL whole blood over 28 days, 82.8 mL of which is taken during the first 10 days. If more than 2 patients withdraw from the study without giving more than 12 samples, the withdrawing patients will be replaced. Outpatients who consent to the standard pharmacokinetic study will be admitted in the hospital for the first 3 days.

Population Pharmacokinetics (Sparse Sampling)

Each of remaining patients (approximately 35 per arm), not enrolled in the rich pharmacokinetic sub-study, contribute to the population pharmacokinetic study, which consists of 13 sampling points (see Multimedia Appendix 2, page 15). Seven of the 13 time points coincide with the timing of the sample for membrane feeding (including the baseline sample), and thus do not require an extra venepuncture (ie, days 0, 2 [52 hrs; 4 hrs after last dose of ivermectin], days 7, 10, 14, 21 and 28). Six of the 13 time points are specific for the population pharmacokinetic study and will require an extra venepuncture (50, 54, 60, 72, 96 and 120 hours, ie, 2, 6, 12, 24, 48, and 120 hrs after the third and last dose of ivermectin). To ensure an equal distribution of samples across the different sampling time points for the extra 2 visits, participants will be divided into 4 extra sampling groups; each of which will contribute 2 extra time points, with the exception of group B which will contribute 1 extra time point (Table 3). In addition, a maximum of 4 finger pricks (0.5 mL whole blood) will be taken at days 2 plus 4 h, 7, and at each of the 2 population pharmacokinetic visits. Thus the total number of samples per participant will be 13 and involve a total of 46.4 mL of whole blood (including the 7 samples for the main trial). The sampling times will be noted in the case record form (CRF), and the patient given a reminder card to return to clinic at their allocated time.

Table 3. Schedule of extra sampling points for population pharmacokinetic study by 4 sampling groups.

Subject Group	Sample day[a] (plus hours after 3rd ivermectin dose)	Sample absolute time[a], hours	Number per sampling strata
A	2.08 (+2 hours)	50	9
	2.25 (+6 hours)	54	
B	2.25 (+6 hours)	54	8
C	2.50 (+12 hours)	60	9
	3 (+24 hours)	72	
D	4 (+48 hours)	96	9
	5 (+72 hours)	120	
Total			35

[a]Extra visits that need to be made specifically for the population pharmacokinetic samples. The other 7 visits contributing to the population pharmacokinetic analysis (days 0, 2, 7, 10, 14, 21, 28) coincide with the scheduled visits in the main trial. The first day is day=0; day 1 starts 24 hours after the first dose. The allocation to the sampling strata will be at random. However, if a participant indicates he/she is not able to attend a certain follow-up day, the strata can be replaced by another sampling schedule (within the same allocation strata, eg, for BMI, gender, etc) until all 15 or 16 allocations per sampling group have been used.

In anticipation of a 40% refusal rate or loss to follow-up, we estimate that the combined rich and population pharmacokinetic sub-studies will contribute 361 samples including 47 baseline samples (100%) and 314 (60%) follow-up samples out of a potential 524 follow-up samples across 22 sampling time points after baseline, 20 of which overlap, with a total of 12 to 47 observations per time point (see Multimedia Appendix 2,Table 2, page 15).

Laboratory Procedure

Mosquito Colonies

See the “Procedures for Assessing Efficacy and Safety Parameters” section above for use of mosquito colonies and procedures to assess the primary (mosquito survival) and secondary entomological endpoints (sporogony). The section below describes the maintenance of the mosquito colonies.

The mosquito colony used in this study will be *An. gambiae s.s.* Kisumu strain, originally from Kisumu, Kenya. The strain is maintained at the KEMRI/Centre for Global Health Research (CGHR) insectaries and is susceptible to all insecticides approved by the World Health Organization (WHO). When performing membrane feeds on infected human blood, mosquitoes will be kept and fed in cages or paper cups. The cages or paper cups will be kept in a temperature- and humidity-controlled insectary. The feeding and the storage of live infected mosquitoes will occur in sealed rooms with at least 2 doors and/or barriers separating the inner rooms from the outside. Mosquitoes will not be removed from their enclosures, with exception of the cage for oocyst determination. During transportation, live infected mosquitoes will be transported within paper cups that are covered with a moist towel and enclosed within locked cool-boxes to remove any chances of escape. The cool-boxes will only be opened within the confines of a double door insectary.

Ivermectin Plasma Concentration

The LC50 has been previously estimated using spiked blood (blood to which known concentrations of ivermectin are added) in membrane feeding assays [12,15]. We will test the concentration of ivermectin in human plasma in order to provide data for a pharmacokinetic/dynamic analysis to obtain estimates

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of the 10-day LC50 and time post-treatment that the transmission blocking effects (on mosquito survival and oocyst rates) lasts.

Hemoglobin Testing

Hb will be tested using HemoCue (Angelholm, Sweden) photometers.

Thick and Thin Blood Smears for Malaria

Thick and thin blood films for parasite counts will be obtained and examined. Malaria parasites will be counted against 200 high power fields before a slide is declared negative [39].

Processing of Pharmacokinetic Samples

Plasma will be stored locally on site at -20°C or in liquid nitrogen and shipped to a central laboratory for storage at -70°C prior to batch analysis at the Liverpool School of Tropical Medicine. Samples will be shipped in dry ice to the laboratories in Liverpool, United Kingdom where the plasma concentrations of ivermectin and piperaquine will be determined using assays validated to international Food and Drug Administration (FDA) standards. Plasma concentration-time data will be used to evaluate pharmacokinetic parameters including CL/F (oral clearance), V/F (oral volume of distribution), and Ka (absorption rate constant) using population pharmacokinetic methods. Area under the curve (AUC) and half-life will also be calculated.

Statistical Methods

A study statistical analytical plan for the final analysis, that supersedes the study protocol, has been drawn up during the course of the study before the unblinding of data at database lock (see Multimedia Appendix 3).

Procedures for Assessing Efficacy and Safety Parameters

Membrane Feeding Procedure

The following procedures will be conducted in accordance with a standard membrane feeding protocol [40]. A 1 mL sample of the participant's blood will be drawn into a sodium heparin (coated) tube pre-heated to 37.5°C. Within 2 minutes the blood will be placed in a glass bell membrane feeding system and cups of mosquitoes will commence feeding. For each feeding 3 new cups (2 cups for mosquito survival, and 1 cup for oocysts) of 50, 3 to 5 day old female, insectary-reared *An. gambiae s.s.* mosquitoes will be presented to the membrane feeder for 20 minutes. The number of mosquitoes with an engorged abdomen will be counted and those with lean abdomens discarded. Each day up to day 28 (mosquito survival cups) or day 10 (oocyst cup), the number of dead mosquitoes will be counted and removed. After the initial feeding on human blood, the mosquitoes will be kept in an incubator and maintained on sugar feeds. Insectary staff assessing mosquito survival and oocyst development will be blinded to all characteristics of the cups (ie, participant identification, study arm, duration between treatment and feeding, and feeding method).

Primary Efficacy Outcome

The primary outcome will be the survival of mosquitoes (from the 2 mosquito survival cups) at 14 days after feeding on blood taken from study participants who started the 3-day ivermectin and DP regimen 7 days earlier.

Secondary Efficacy Outcomes

Although the primary endpoint is assessed at day 14, the study will collect survival data of mosquitoes at each day up to day 21 or 28 for the mosquito survival cups and day 10 in the case of oocyst cups, after each feeding experiments performed at 0, 2 days plus 4h, 7, 10, 14, 21, 28 days after start of treatment. The methods will be identical to that described for the primary outcome where each day beyond day 14 the number of dead mosquitoes will be counted and removed until day 28 inclusive. The exact number of follow-up days (21 or 28 days) will be subject to logistical constraints of the laboratory, and mortality rates in the mosquito populations which will be further determined prior to the start of the study. The aim is to determine the median time to mortality, which requires that at least half of the mosquito population has died in each arm. It is anticipated that 21 days will be sufficient.

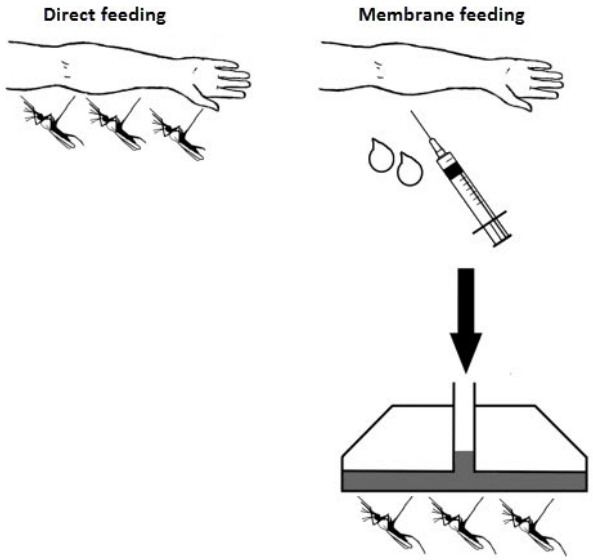
Direct Skin Feeding and Mosquito Survival

A sub-study will determine the effect of direct feeding versus membrane feeding on mosquito survival, after feeding experiments performed at 7 days after the start of treatment. In direct skin feeding assays, 1 cup of 50 mosquitoes is placed directly on the skin of the human host and allowed to feed for 15 minutes (Figure 3). Further procedures after direct feeding are identical to those after membrane feeding.

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FOI 26-3132 - Document 5

Figure 3. Difference between membrane feeding and direct feeding (adapted from Bousema et al 2012 [38]).



Infectivity to Mosquitoes (Oocyst Polymerase Chain Reaction)

On day 10 post membrane feeding, when residual DNA from the blood meal is highly unlikely [14,41,42], all surviving mosquitoes in the oocyst cup will be preserved to determine oocyst prevalence by PCR. Mosquitoes will be homogenized and processed, in 2 pooled batches per cup.

Asexual Treatment Response and Parasite Clearance

Standard methods will be used to assess the in vivo treatment response to DP using the microscopy and rapid diagnostic test (RDT) data collected at each scheduled follow-up visit and criteria described by World Wide Antimalarial Resistance Network (WWARN) [43].

Pupillometry

In animal studies, mydriasis has been shown to be a first sign of ivermectin toxicity. To monitor for possible toxicity, pupil diameter size will be measured at baseline and each scheduled visit using a portable, single-button activation, battery operated hand-held pupillometry device that very accurately measures pupil size requiring no calibration (NeurOptics VIP-200 Variable Pupillometer). This device measures the pupil 30 times per second over a 5-second period and provides the average pupil diameter and standard deviation (+/- 0.1 mm). The measurements will be taken in a dark room with standardized lighting conditions.

Electrocardiogram Monitoring

Piperaquine can potentially lead to prolongation of the rate corrected QT interval (QTc) on an electrocardiogram (ECG). To exclude a possible interaction between ivermectin and piperaquine leading to an increased QTc interval, 12-lead ECGs will be performed to measure the QTc interval at baseline, day 2 pre last dose, day 2 at 4 to 6h post last dose and again at day 28. The day 28 sample is included as it can be difficult to assess a true baseline in patients with acute malaria, as malaria and fever are known to increase the heart rate and decrease the QTc interval. On day 28 most, if not all, patients will be malaria free and residual piperaquine levels low enough not to affect QTc intervals. A portable ECG machine (MAC 600, General Electric, US) will be used with automated ECG interpretation. Patients with a QTc value of 480 ms or greater prior to the last dose of DP will not receive the last dose of DP, but receive a full course of artemether lumefantrine instead. Fridericia's correction will be used to calculate the QTc values for final data analysis using the following equation: $QTc = QT/RR^{0.33}$.

Adverse Events

Adverse events and serious adverse events will be monitored, managed, and recorded during the course of the study. They will be recorded and tabulated for each treatment arm, overall, and per body system (see Multimedia Appendix 2, Section 9.6, Safety Monitoring and Reporting).

Ethics Approval and Consent to Participate

This protocol, the informed consent documents, and patient information sheets have been reviewed and approved by the Research Ethics Committees at KEMRI (protocol #2775), LSTM (protocol #14.002), and JOOTRH. The Centers for Disease Control and Prevention (CDC, protocol #6720) gave approval for reliance on the KEMRI institutional review board (see Multimedia Appendix 4 Ethics Approvals KEMRI, CDC, LSTM, and JOOTRH).

Results

Recruitment started July 20, 2015. Enrolment was completed May 2016, and clinical follow-up was completed 4 weeks later in June 2016. Mosquito follow-up was completed in July 2016, 4 weeks after completion of the clinical follow-up. Unblinding and analysis will commence once the database has been completed, cleaned, and locked.

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Discussion

Principal Findings

New strategies for malaria control, and eventually for elimination are critically needed. This study will seek to answer the question as to whether higher doses of ivermectin (300 and 600 mcg/kg/day for 3 days) are well tolerated, safe, and result in longer durations of mosquitocidal effects than standard 150 to 200 mcg/kg single dose treatments. This study requires major infrastructure and collaboration, as it brings together the disciplines of clinical medicine, entomology, parasitology, pharmacokinetics, and pharmacogenetics in a clinical trial. For this study, 141 patients and 150,000 mosquitoes will each be followed for 28 days. For this reason, this trial has been placed at the KEMRI, CDC, and LSTM collaboration in western Kenya, a research site, which in collaboration with its partners, has been conducting research for over 35 years and has the capacity to undertake such a trial. An important possible limitation of this study is that it will enroll participants with symptomatic malaria, whereas possible future applications of high-dose ivermectin may involve MDA with ACT's targeting asymptomatic carriers and uninfected individuals in addition to symptomatic patients. Should this study show promising results, then the next step will be to evaluate safety, tolerability, and efficacy in younger age groups with the ultimate goal of testing its effect on malaria transmission when applied at the population level through MDA.

Conclusion

High-dose ivermectin, if found to be safe and well tolerated, could potentially complement existing tools for malaria elimination.

Acknowledgments

Many thanks to the members of the Trial Steering Committee (Teun Bousema, Kevin Kobylinski, and Brian Foy) and the Data Monitoring and Ethics Committee (Alejandro Krolewiecki, James Oloo, Timothy Collier, and Carlos Chaccour). Further thanks to the Mundo Sano Foundation and Elea Laboratories for donating the ivermectin, and NeurOptics for donating pupillometers. Finally, we would also like to thank Dr Juliana Otieno, Medical Superintendent, for accommodating the study at JOOTRH; Donald Tjia, Ophthalmologist, for supporting the study; and Jacco Veldhuyzen, Emergency Medicine Physician, for assisting in interpreting ECGs. This paper is published with the permission of KEMRI Director.

This study is funded by the Malaria Eradication Scientific Alliance (MESA), through a sub-grant from the Bill and Melinda Gates Foundation (BMGF). Neither MESA nor the BMGF has or will have any role in the design of the study, the collection, analysis, and interpretation of data, or in the writing the manuscript.

Authors' Contributions

FtK and MS conceived the study. MS, PPH, and FtK wrote the grant. MS, EO, and FtK drafted the protocol. DW provided statistical expertise and verified the sample size calculation. GA and SW conducted the Monte Carlo simulations to define the dosing regimen and further developed the pharmacokinetic sub studies. All investigators contributed to the refinement of the study protocol and approved the final version. MS and FtK drafted the manuscript. All authors read and approved the final manuscript prior to submission. The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Conflicts of Interest

None declared.

Multimedia Appendix 1

Summary of study design and schedule of assessment (SPIRIT flow diagram).

[PDF File (Adobe PDF File), 288KB-Multimedia Appendix 1]

Multimedia Appendix 2

Full study protocol (including SPIRIT checklist): v4.1, dated 14-Jan-2016.

[PDF File (Adobe PDF File), 3MB-Multimedia Appendix 2]

Multimedia Appendix 3

Statistical Analytical Plan (SAP): v1.0, dated 19-Feb-2016.

[PDF File (Adobe PDF File), 347KB-Multimedia Appendix 3]

Multimedia Appendix 4

Ethics approvals v4.1: KEMRI, CDC, LSTM, and JOOTRH.

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[PDF File (Adobe PDF File), 748KB-Multimedia Appendix 4]

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Abbreviations

ACT: artemisinin-based combination therapy
AUC: area under the curve

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CDC: Centers for Disease Control and Prevention
Cmax: maximum drug concentration
CYP: cytochrome P450
CYP3A4: cytochrome-P450 3A4
DHA: dihydroartemisinin
DP: dihydroartemisinin-piperaquine
ECG: electrocardiogram
Hb: hemoglobin
ICC: intracluster correlation coefficient
JOOTRH: Jaramogi Oginga Odinga Teaching and Referral Hospital
KEMRI: Kenya Medical Research Institute
LC50: lethal concentration 50%
LLINS: long-lasting insecticide treated nets
LSTM: Liverpool School of Tropical Medicine
MDA: mass drug administration
PCR: polymerase chain reaction
RDT: rapid diagnostic test
QTc: rate corrected QT interval on an electrocardiogram

Edited by G Eysenbach; submitted 06.09.16; peer-reviewed by CJ Chaccour, H Slater, T Bousema, RW Steketee; comments to author 04.10.16; revised version received 17.10.16; accepted 18.10.16; published 17.11.16

Please cite as:
Smit MR, Ochomo E, Aljayyoussi G, Kwambai T, Abong'o B, Bayoh N, Gimnig J, Samuels A, Desai M, Phillips-Howard PA, Kariuki S, Wang D, Ward S, ter Kuile FO
Efficacy and Safety of High-Dose Ivermectin for Reducing Malaria Transmission (IVERMAL): Protocol for a Double-Blind, Randomized, Placebo-Controlled, Dose-Finding Trial in Western Kenya
JMIR Res Protoc 2016;5(4):e213
URL: http://www.researchprotocols.org/2016/4/e213/
doi: 10.2196/resprot.6617
PMID: 27856406

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Veerapaneni Spoorthi, Surapaneni Sasank. Utility of Ivermectin and Doxycycline combination for the treatment of SARS-CoV-2. IAIM, 2020; 7(10): 177-182.

Original Research Article

Utility of Ivermectin and Doxycycline combination for the treatment of SARS-CoV-2

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International Archives of Integrated Medicine, Vol. 7, Issue 10, October, 2020.
Available online at http://iaimjournal.com/
ISSN: 2394-0026 (P) **ISSN: 2394-0034 (O)**
Received on: 02-10-2020 **Accepted on:** 10-10-2020
Source of support: Nil **Conflict of interest:** None declared.
How to cite this article: Veerapaneni Spoorthi, Surapaneni Sasank. Utility of Ivermectin and Doxycycline combination for the treatment of SARS- CoV-2. IAIM, 2020; 7(10): 177-182.

Abstract

Background: On March 11, 2020, the World Health Organization (WHO) officially declared coronavirus disease 2019 a global pandemic. Ivermectin and Doxycycline against SARSCoV-2 under in-vitro conditions showed beneficial results. However, large population studies with this combination are not elaborately studied, which was the aim of our study.
Materials and Methods: A total of 122 patients admitted in a tertiary care centre, who tested positive for Reverse-transcriptase-polymerase-chain-reaction (RT-PCR) for SARS-CoV2 were included in the study and a total sample size of 100 patients was obtained after exclusion. 50 patients of the treatment group were treated with Ivermectin-Doxycycline combination.
Results: The results had shown a significant reduction not only in mean duration of hospital (3.70 ± 2.27 days vs 4.69 ± 2.3 days), but also in complete resolution of symptoms stay (6.67 ± 2.01 days vs 4.69 ± 2.3 days). In a small subset of 10 patients RT-PCR for COVID was tested on 10th day after the symptom onset in both the groups and there was no difference to be found. There was no significant difference in the side effect profile of either groups.
Conclusion: Our study supports the benefits of utilization of combination of Doxycycline and Ivermectin in mild to moderate COVID-19 infection in terms of early recovery based on the time for symptom resolution and the mean duration of hospital stay.

Key words

COVID19, SARS-CoV2, Ivermectin-Doxycycline, RT-PCR.

Veerapaneni Spoorthi, Surapaneni Sasank. Utility of Ivermectin and Doxycycline combination for the treatment of SARS-CoV-2. IAIM, 2020; 7(10): 177-182.

Introduction

After the origin of novel coronavirus (2019-nCoV) in December 2019 at Wuhan, On March 11, 2020, the World Health Organization (WHO) officially declared coronavirus disease 2019 a global pandemic, the prevalence of this virus had shown an exponential growth throughout the world [1].

Though Hydroxychloroquine was initially tried and multiple small trials showed marginal benefit, a recent meta-analysis showed that Hydroxychloroquine does not appear to offer significant benefits in terms of reducing the viral load, resolution of radiological findings, or progression of the COVID-19 and Hydroxychloroquine might be associated with increased all-cause mortality [2].

Recent study on Ivermectin against SARSCoV-2 under in-vitro conditions revealed that it can inhibit viral replication. The single treatment of this drug was able to reduce the SARS-CoV-2 virus up to 5000-fold in Vero-hSLAM cells bathed with Ivermectin within 48 hours. However, no further reduction was reported with further increase in time period i.e. up to 72 hours. Moreover, no toxicity was seen with the drug at any point of time [3]. Doxycycline acts on Zinc as part of their MMP complex, chelates it and also acts via reduction in pro-inflammatory IL-6 levels, hence may reduce the viral load [4].

Ivermectin-Doxycycline combination showed a trend toward superiority to the Hydroxychloroquine-Azithromycin combination therapy in the case of patients with mild to moderate COVID19 disease [5].

Materials and methods

A total of 100 patients admitted in a tertiary care centre, which tested positive for RT-PCR for SARS-CoV2 were included in the study.

Patients were seen at baseline for enrollment, initial data collection and treatment at day-0, and again for daily follow-up. Each day, patients received a standardized clinical examination and all clinical data were collected using standardized questionnaire.

Study variables collected on each patient included the following; patient demographics: age, gender; clinical characteristics: admission date, discharge date, length of stay (LOS), day of symptom resolution; comorbidities including: chronic lung disease, hypertension, asthma, chronic obstructive pulmonary disease (COPD), diabetes mellitus, immunodeficiency, and cancer (defined as active or past/ resolved). Additionally, intensive care unit (ICU) status and ventilator use at any point during admission, minimum O2 saturation level collected on day of admission in the emergency department. The duration and dosages of all therapies for COVID-19 were collected.

This prospective study was aimed to establish the efficacy of combination of Ivermectin and Doxycycline in the treatment of mild to moderate Covid-19 in India.

To study the outcome of the above mentioned drugs in at least 50 patients of covid-19 in comparison with the patients of covid-19 administered with placebos (Vitamin B6).

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Inclusion criteria

- All patients diagnosed with Covid-19 by RT-PCR, with mild to moderate symptoms.
- Respiratory Rate < 24/min and SpO2 > 93% on room air.
- Absence of Oxygen support on admission.
- Duration of symptoms prior to admission ≤ 7 days.

Exclusion criteria

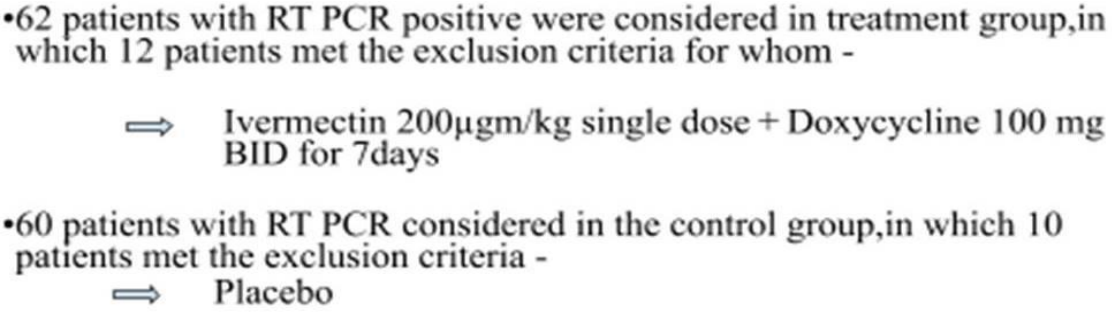
- Patients with a history of allergy to Ivermectin or Doxycycline
- Pregnant or lactating women.

Veerapaneni Spoorthi, Surapaneni Sasank. Utility of Ivermectin and Doxycycline combination for the treatment of SARS-CoV-2. IAIM, 2020; 7(10): 177-182.

- Patients with a history of chronic liver disease (SGPT > 3 times of normal value).
- Patients with a history of chronic kidney disease (eGFR <60 ml/min/1.73 m^2).
- Patients with a history of chronic heart disease.

This study was done on 122 patients taken as a study population (**Figure – 1**) and Ethical committee clearance was obtained.

Figure – 1: Group distribution.



Statistical analysis

The quantitative data were described as the mean ± standard deviation, or as the median (min − max). The qualitative data were described by number of cases (proportion, %). Patient characteristics were compared using the χ^2 test or Fisher's exact test for categorical data, and the Wilcoxon rank-sum test or Student's *t* test for continuous data. Potential influencing factors of viral clearance were analyzed by univariate and multivariate Cox regression models. A p value lower than 0.05 was required for statistical significance. All of the analysis was performed using SPSS Version 22.0.

Results

Out of 62 patients in the treatment group, 6 patients had a history of chronic kidney disease, out of which 3 were on Hemodialysis and all the patients had e GFR < 30 ml/kg/min/m^2, 2 antenatal patients along with 1 lactating patient, 2 had congestive heart failure and 1 had decompensated chronic liver disease with severe transaminitis.

Out of 100 patients, male population was slightly higher than the female population in both the groups, diabetics were slightly more in the placebo than in the treatment group. Very small number of patients had pulmonary issues, which were almost equal in both the groups (**Table – 1**).

The mean duration of hospital stay was 6.67 ± 2.01 days in treatment Group and 7.89 ±2.35 days and the entities were compared using unpaired t-test, which attained statistically significant (P = 0.01) as per **Figure – 2** and **Table - 2**.

Table – 1: Baseline characteristics of patients.

Patient baseline characteristics	Treatment group	Placebo group
Age (Median ± SD)	50.95 +_13.64	48.72+_13.42
Males	26 (52%)	28(56%)
Females	24(48%)	22(44%)
Diabetics	6	7
Hypertensive	5	4
Old pulmonary tb	1	1
Chronic obstructive lung disease	2	2

Veerapaneni Spoorthi, Surapaneni Sasank. Utility of Ivermectin and Doxycycline combination for the treatment of SARS-CoV-2. IAIM, 2020; 7(10): 177-182.

Figure – 2: Hospital stay and symptoms duration.

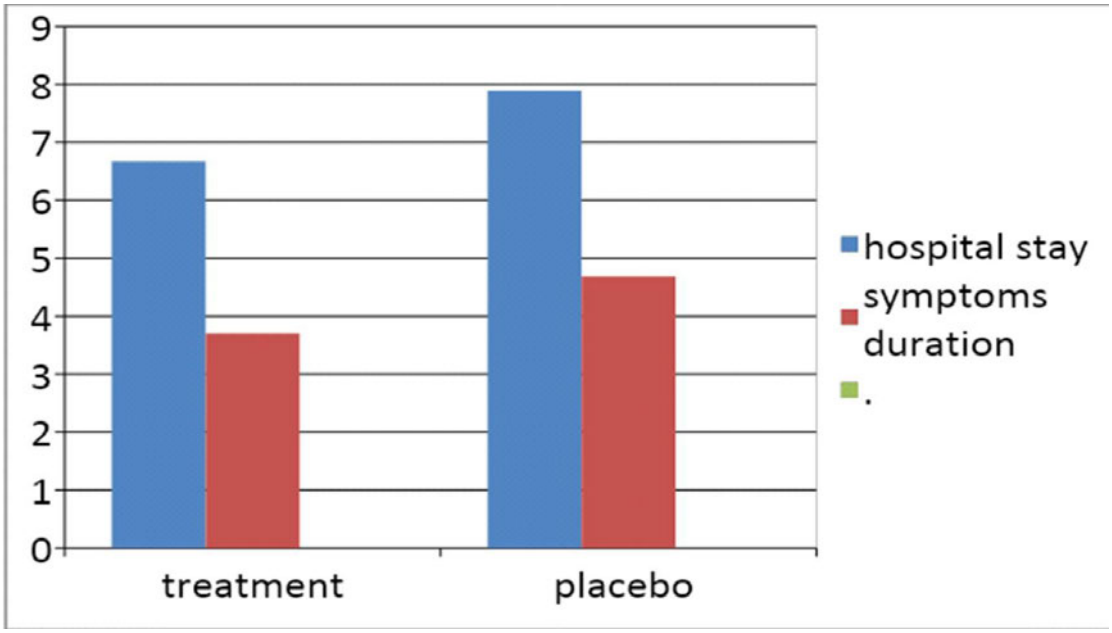


Table – 2: Hospital stay and symptoms duration.

	Mean +_SD	Mean +_SD	P value
Mean duration of hospital stay (days)	6.67 ± 2.01	7.89 ±2.35	0.01
Time for symptom resolution (days)	3.70 ±2.27	4.69 ±2.3	0.03

Table – 3: Side effects.

Side effects	Treatment group	Placebo group
Diarrhea	4	2
Vomiting	3	2
Pruritus	1	0

The duration for complete resolution of symptoms was 3.7 ± 2.27 days in the treatment Group and 4.69±2.3 days and a similar comparison between the either groups obtained statistically significant (P = 0.03). Comparison of side effects in both groups was as per **Table – 3**.

Discussion

Our findings are consistent with the benefits of utilization of combination of Doxycycline and Ivermectin in mild to moderate COVID-19 infection. The results have shown a significant reduction not only in mean duration of hospital (3.70 ± 2.27 days vs 4.69 ±2.3 days), but also in complete resolution of symptoms stay (6.67 ± 2.01 days vs 4.69 ± 2.3 days). These results were consistent with another study done at Bangladesh, where mean resolution of symptoms were around 5.93 days [6].

Compared to the prior studies, the cohort of our patients was elderly, with slight male predominance. The previous studies done similarly had a slightly younger age group of population.

In a small subset of 10 patients RT-PCR for COVID was tested on 10th day after the symptom onset in both the groups, there was no difference, with RT PCR being positive in 2 members of either groups on day 7 or positive in a single individual of each group on day 10. A recent multi-centric study compared to 1,918 conventionally treated patients, a survival benefit for Ivermectin mortality rate was observed. In the same study, the mean duration of symptom

resolution was reduced by 7.1 ± 2.1 days compared to those without Ivermectin. However, this study also included patients with respiratory failure [7].

Tetracyclines may be effective agents in the treatment of Covid-19 due to their ability to chelate Zinc compounds on matrix metalloproteinases (MMP) on which coronaviruses rely heavily for survival, cell infiltration, cell to cell adhesion and replication, many of which has Zinc as part of their MMP complex [3]. Ivermectin destabilizes the Impα/β1 heterodimer, via which the host cell antiviral response is reduced [8, 9].

In our study, the side effects of these drugs were reviewed and the common side effects included GI disturbances seen in 4 patients, however with the administration of proton pump inhibitors and regularizing the timing of the antibiotic after food intake, these side effects reduced after 2 days .1 patient had pruritus relieved with antihistamines .One patient in the treatment group expired after deteriorating on 5th day after he developed coagulopathy.

Our study had a few limitations, first the size of our sample was smaller, consisting of 100 patients only. Second all the patients could not be tested for RT PCR on the 10th day of symptoms, as most of them were discharged after symptomatic relief i.e., after around 7 days of admission.

Third, we could not consider patients with atypical covid symptoms such as diarrhea, acute gastritis [10] as we could not categorize them based on severity.

Conclusion

Our study supports the benefits of utilization of combination of Doxycycline and Ivermectin in mild to moderate COVID-19 infection in terms of early recovery based on the time for symptom resolution and the mean duration of hospital stay.

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FOI 26-3132 - Document 5

International Journal of Infectious Diseases 122 (2022) 553–558

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

COVID-19 prophylaxis with doxycycline and zinc in health care workers: a prospective, randomized, double-blind clinical trial

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[14] *Details of "OD-doxy-PNV-COVID-19 Trial" investigators are given in the Appendix*

ARTICLE INFO

Article history:
Received 2 March 2022
Revised 26 April 2022
Accepted 13 June 2022

Keywords:
Doxycycline
Zinc
Placebo
COVID-19 infection
Prophylactic

ABSTRACT

Objectives: This study aims to assess the efficacy of a combination treatment of doxycycline and zinc in the primary prevention of COVID-19 infection in Tunisian health care workers compared with two control groups.

Methods: We conducted a prospective, randomized, double-blind clinical trial over 5 months to determine the efficacy of a preventive combination treatment dose of doxycycline (100 mg/day) and zinc (15 mg/day), compared with a single-dose treatment with doxycycline versus placebo. The effectiveness of preventive treatment was measured by the significant decline in the number of cases of COVID-19 infection and/or a decrease in the viral load as determined by SARS-CoV-2 cycle threshold value using reverse transcription polymerase chain reaction tests.

Results: We detected a significant decrease of SARS-CoV-2 infection in the group that received both doxycycline and zinc compared with other participants. We also demonstrated that COVID-19 infection was neither associated with diabetes ($P = 0.51$) nor associated with hypertension ($P = 0.99$), asthma ($P = 0.52$), and chronic obstructive pulmonary disease ($P = 0.27$).

Conclusion: Our findings indicated that preventive therapy reduced the risk of SARS-CoV-2. These results suggest that the combination of doxycycline and zinc has a protective effect in patients with SARS-CoV-2 infection.

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https://doi.org/10.1016/j.ijid.2022.06.016
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Introduction

Prevention of COVID-19 transmission and infection includes nonpharmacologic intervention and specific protection through chemoprophylaxis or immune prophylaxis (Agrawal et al., 2020). Since the 1970s, the protective efficacy of doxycycline has been demonstrated in the prophylaxis of malaria for travelers to endemic areas (Tan et al., 2011). In Tunisia, doxycycline has been widely used in the prevention of malaria for military personnel going on missions to endemic areas (Ajili et al., 2013). It has a low cost and a favorable safety profile and has been proposed as a treatment for COVID-19 (Malek et al., 2020). Doxycycline has been used as a curative treatment for COVID-19 in Brazil and India (Butler et al., 2021), whereas in the UK, it is recommended for suspected COVID-19 pneumonia in patients who are at high risk for adverse outcomes or where bacterial infection is suspected (Butler et al., 2021).

Zinc also has antiviral effects against certain viruses (Guo et al., 2004). In fact, zinc supplementation improves the mucociliary clearance, strengthens the integrity of the epithelium, decreases viral replication, preserves antiviral immunity, attenuates the risk of hyperinflammation, supports antioxidative effects, reduces lung damage, and minimizes secondary infections (Wessels et al., 2020).

The purpose of this study was to evaluate the efficacy and safety of doxycycline with or without zinc in preventing COVID-19 infection. The study focused on Tunisian health care workers (HCWs), and the outcome measures were the decreased number of infected cases with COVID-19 and/or the decrease in the viral load, as determined by the cycle threshold (Ct) values of the reverse transcription polymerase chain reaction (RT-PCR) tests for SARS-CoV-2.

Materials and methods

Patients and methods

We conducted a prospective, randomized, double-blind clinical trial for 5 months (from November 12, 2020, through February 10, 2021). The study was conducted at different military sites of investigation (Military Hospital of Tunis, Military Hospital of Bizerte, Military Hospital of Gabes), was registered in the clinical trial database (NCT04584567), and approved by the local ethical committee of the General Directorate of Military Health.

The study included consenting HCWs, aged between 20 and 65 years, who did not have signs or symptoms of respiratory infection (cough, fever >38.0°C, difficulty breathing, shortness of breath, chest pains), or other symptoms associated with COVID-19, such as faintness, myalgia, headaches, and nausea or vomiting.

The study excluded HCWs with positive SARS-CoV-2 RT-PCR results or positive SARS-CoV-2 serum tests (IgM or IgG). Positive SARS-CoV-2 RT-PCR was determined by a cycle threshold (Ct) value below 33, using the Xpress SARS-CoV-2 kit (Xpert, Cepheid, Sunnyvale, CA). The GeneXpert is a system that automates and integrates sample preparation, nucleic acid extraction, amplification, and detection of the target sequences using real-time RT-PCR assays. The real-time RT-PCR reagents, including primer N2, target the virus nucleocapsid phosphoprotein (N) gene for specific detection of SARS-CoV-2 and human nucleic acid. The Cepheid Xpert Xpress SARS-CoV-2 kit detects N2 and E gene, and solely N2 indicates positive results. The presence of only E indicates presumptive positive results, whereas the presence of only A Sample Processing Control (SPC) indicates negative results. The absence of all markers indicates an invalid result (Moran et al., 2020).

Ct is a value that can broadly classify the concentration of viral genetic material as low, medium, or high in a patient sample, using RT-PCR test. It tells us approximately how much viral genetic material is in the sample. A low Ct indicates a high concentration of viral genetic material, which is associated with high risk of infection. A high Ct indicates a low concentration of viral genetic material, which is associated with a lower risk of infection.

For the qualitative detection of antibodies against SARS-CoV-2, we used the Biosynex COVID-19 Ag+ BSS test (Illkirch-Graffenstaden, France).

Additionally, we excluded HCWs who had a known hypersensitivity to doxycycline or zinc. HCWs with comorbidities (gastric bypass, epilepsy, cardiovascular disease, renal failure) or being treated with vitamin A during the study were excluded. Women who were pregnant or nursing were also excluded.

Eligible participants were randomized in three parallel groups as follows:

Group A: doxycycline (100 mg/ day) and zinc (15 mg/ day) combined orally for 6 weeks.

Group B: doxycycline (100 mg /day) orally for 6 weeks.

Group C: placebo orally for 6 weeks.

Allocated study groups were randomly assigned by an interactive web response system (Dacima), using simple 1:1 nonstratified sequence and a block size of six. Allocation sequences are set automatically by the interactive web response systems software.

Doxycycline was manufactured by Philadelphia Pharmaceutical (batch number 12927222). Placebo was also produced by the same manufacturer (Philadelphia Pharmaceutical). Placebo contains the same excipients and no active substances. Zinc (zinc bisglycinate) was manufactured by Albion human nutrition (batch number F001).

The participants were screened 72 hours before enrollment. They were examined physically, tested for SARS-CoV-2 RT-PCR and serum test (IgM/IgG). All personal data were anonymized, and signed written informed consent forms were obtained before the beginning of the study. The participants were monitored at days 21, 42, and 49 after enrollment. At each follow-up visit, participants were asked about their medical compliance and drug safety, underwent a clinical examination, and had an RT-PCR with calculated Ct values. After screening for patient's eligibility, a balanced randomization was done on the same day by the investigator. Allocated study drug was provided to the patient by the Military Hospital Pharmacist, according to the allocation details provided by the interactive web response systems. The patient received the drug on the same day of the randomization. Study blindness was maintained by a blind allocation, and drugs bottles were labeled anonymously by the pharmacist. Statistical analysis of the database was performed with blinded groups. Unblinding was performed at the final statistical report review. The Dacima Clinical Suite software (Montréal, Québec, Canada) was used to collect the data and to randomize participants on the basis of the Food and Drug Administration 21 Code of US Federal Regulations part 11, Health Insurance Portability and Accountability Act, and International Conference on Harmonization requirements.

Study end points

The first end point of this study was to assess how doxycycline with low dose can be used as a preventive treatment to decrease the number of cases of COVID-19 in the active arms compared with the placebo. Participants for each randomized treatment arm (doxycycline-only or combined with zinc) were compared with placebo. The second end point was intended to quantify by RT-PCR the number of infected cases with COVID-19 and the viral ribonucleic acid load after each treatment.

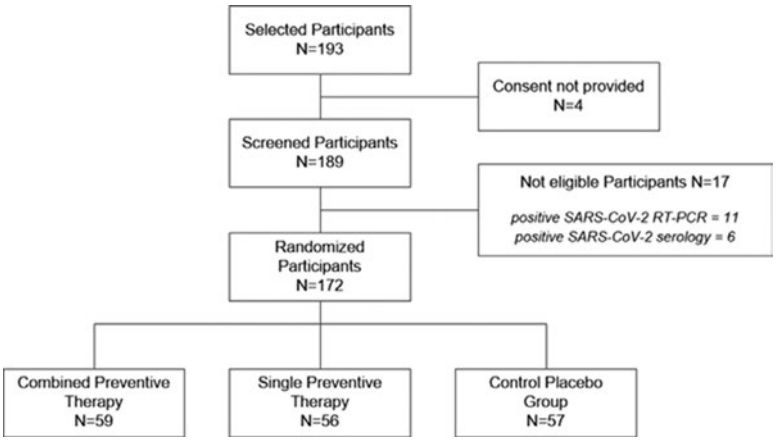


Figure 1. Design and analysis of the study. A total of 172 eligible participants were randomized into the three study arms: 59 in the combined doxycycline and zinc arm, 56 in the doxycycline-only arm, and 57 in the placebo arm. RT-PCR, reverse transcription polymerase chain reaction.

Sample size

Sample size was estimated by the Cochran-Armitage test for linear trend in proportions. Considering "a sample power of 80%", an expected proportion of the primary end point of 8% in group A, 10% in group B, and 14% in group C, with a confidence level of 95%. The expected sample size was estimated up to 1100 overall eligible subjects. However, due to the pandemic situation and the rush for nationwide vaccination against the SARS-CoV-2, the study was ended after including 172 randomized patients.

Evaluation

During the follow-up period (6 weeks), we monitored the SARS-CoV-2 infection by RT-PCR tests at days 21, 42, and 49. The last evaluation was done by day 49 to determine if a potential infection occurred after the treatment stopped at day 42.

Statistical analysis

For statistical analysis, qualitative data were described by frequency and percentage of valid values. Continuous variables were used to describe mean and standard deviation (SD). Chi-square, Mann-Whitney *U* test, and Kruskal-Wallis tests (one-way analysis of variance) were used to make inferential comparisons, with a 5% significance level as a threshold. Differences were considered significant if $P \leq 0.05$. No further statistical analysis was performed due to the study's premature interruption.

Results

From the 193 selected HCWs, 189 were screened. Four subjects declined to participate in the study. The screening phase excluded 17 subjects who were infected with COVID-19: positive SARS-CoV-2 RT-PCR (n = 11) and positive SARS-CoV-2 serology (n = 6). Participants were not symptomatic at the time of RT-PCR/serology testing at inclusion (Figure 1).

The remaining 172 eligible participants were randomized to one of the three study arms: 59 in the combined doxycycline and zinc arm (group A), 56 in the doxycycline-only arm (group B), and 57 in the placebo arm (group C).

The mean age was 38.4 ± 10.7 years (20 to 60 years). In groups A, B, and C, the average age was 38.0 ± 10.6, 38.5 ± 10.0, and 38.7 ± 11.4 years, respectively ($P = 0.934$) (Table 1).

The sex ratio was 1.6 (males = 5, females = 67), with no significant differences between the three study groups ($P = 0.143$). The study arms did not differ significantly for history of influenza (n = 43; 25.0%; $P = 0.927$), hypertension (n = 7; 4.1%; $P = 0.056$), diabetes (n = 4; 2.3%; $P = 0.162$), asthma (n = 2; 1.2%; $P = 0.590$), and chronic obstructive pulmonary disease (COPD) (n = 1; 0.6%; $P = 0.363$) (Table 1).

All participants worked at the Military Hospital of Tunis, which has been active since the beginning of the COVID-19 pandemic. Among the participants, there were different levels of exposure to COVID-19 infection in the workplace, including invasive bedside procedures with patients with COVID-19 (n = 40; 23.3%), intra-operative procedure (n = 19; 11.0%), and routine care (n = 113; 65.7%), with no significant differences between the studied arms ($P = 0.2$).

At the end of the study, a total of 24 subjects tested positive for SARS-CoV-2 (14.0%): 5 (8.5%) in group A, 5 (8.9%) in group B, and 14 (24.6%) in group C, with a significant difference between the three arms ($P = 0.018$) (Figure 2).

In the first group (group A), most participants (4 of 5) were asymptomatic, 1 participant developed symptoms with a persistent fever for 3 days. In the second (doxycycline-only) group (group B), 2 participants were asymptomatic and 3 developed moderate symptoms with fever, headache, and mild nonpersistent cough. In the third (placebo) group (group C), 6 of 14 participants developed symptoms such as fever, cough, and headache. For 2 of them, the cough was persistent, and corticosteroid treatment was prescribed. None of the participants in the three groups required oxygen (Table 2).

This difference was also found from day 21 of the follow-up, when 9 subjects tested positive for SARS-CoV-2 RT-PCR (5.2%), as opposed to 2, 0, and 7 in the A, B, and C groups, respectively ($P = 0.01$). Figure 2 shows the cumulative positive RT-PCR frequency trend among the three groups during the follow-up visits.

The average Ct for participants with a positive SARS-CoV-2 RT-PCR was 22.6 ± 4.7. Comparison between the study arms revealed a significant difference in Ct levels ($P < 0.001$), with the highest mean level of Ct in group A (29.0 ± 1.3), then group B (22.8 ± 4.0), then group C (19.4 ± 2.5). Table 3 shows Ct profiles of the three groups during the study.

We observed that a higher risk of COVID-19 infection was not associated with diabetes ($P = 0.51$), hypertension ($P = 0.99$), asthma ($P = 0.52$), or COPD ($P = 0.27$). Table 4 shows the patient's baseline profile according to SARS-CoV-2 infection. Four of the 24 infected participants had comorbidities. In the combined treatment arm, there were no participants with comorbidities. In the single treatment arm, there was 1 patient with diabetes and 1 with hypertension. In the placebo group, there was 1 participant with

Table 1
Demographic characteristics of the investigated groups.

	All participants N = 172	Group A N = 59	Group B N = 56	Group C N = 57	*P*-value
Age	38.4 ± 10.7	38.0 ± 10.6	38.5 ± 10.0	38.7 ± 11.4	0.936
Gender ratio	1.6 (105 M/67 F)	2.5 (42 M/17 F)	1.2 (31 M/25 F)	1.3 (32 M/25 F)	0.143
Comorbidities, n (%)		14	17	19	
Hypertension	7 (4.1%)	0 (0%)	2 (3.6%)	5 (8.8%)	0.056
Diabetes	4 (2.3%)	0 (0%)	1 (1.8%)	3 (5.3%)	0.161
Asthma	2 (1.2%)	0 (0%)	1 (1.8%)	1 (1.8%)	0.589
COPD	1 (0.6%)	0 (0%)	0 (0%)	1 (1.8%)	0.363
History of COVID-19 infection	0	0	0	0	_
Vaccination		Vaccine not yet available			

COPD, chronic obstructive pulmonary disease; F, female; M, male; NS, not significant.

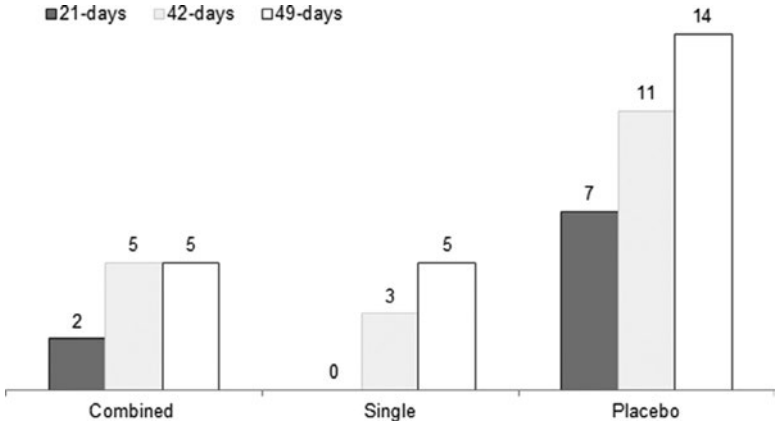


Figure 2. Cumulative positive reverse transcription polymerase chain reaction results for SARS-CoV-2 between the three groups, during follow-up ($P = 0.018$). Each group's histogram represents the number of days during follow-up; 24 days are represented by black, 42 days by gray, and 49 days by white.

Table 2
Clinical symptoms of COVID-19–infected participant.

Symptoms	Combined treatments (Group A)	Single treatment (Group B)	Placebo (Group C)	Total
Asymptomatic	04	02	08	14
Mild to Moderate	01	03	06	10
Total	5	5	14	24

Table 3
Total cycle threshold profile of the three groups during the study.

Study arm	Mean ± SD	95% CI	*P*-value
Combined Therapy	29.0 ± 1.3	27.7-30.3	<0.001
Single Therapy	22.8 ± 4.0	18.6-26.9	
Control Group	19.4 ± 2.5	17.8-21.0	
Overall	22.6 ± 4.7	20.6-24.7	

CI, confidence interval; SD, standard deviation.
Comparison between three groups using Kruskal-Wallis analysis. Results were considered significant if *P*-value <0.05

asthma and 1 participant with COPD. However, in the combined treatment arm, there were no participants with comorbidities.

The safety of the doxycycline/zinc intervention was also assessed during the study follow-up. The most common adverse events reported epigastralgia (n = 14) and nausea (n = 8). In term of side effects, the difference between studied groups were significant only for nausea ($P = 0.032$) but not for epigastralgia ($P = 0.249$) (Table 5).

Discussion

As of January 2022, there are 106 clinical trials underway for prophylactic alternatives to COVID-19, some of which involve antivirals, such as favipiravir or interferons (Ben-Zuk et al., 2021). To the best of our knowledge, this is the only study to evaluate the effect of doxycycline in patients infected with SARS-CoV-2.

In addition to its anti-inflammatory effects, doxycycline has also antiviral activity against several ribonucleic acid viruses *in vitro* (Gendrot et al., 2020). It also chelates zinc from metalloproteases (MMPs) (Sodhi and Etminan, 2020). As a result, their chelating activity may help to inhibit SARS-CoV-2 infection by limiting the virus' ability to replicate in the host, thereby acting as a prophylactic agent (Sodhi and Etminan, 2020).

There have already been some recommendations of doxycycline for COVID-19, particularly in patients with pneumonia and those who are at high risk for complications. There is now evidence of increased use of respiratory antibiotics, including doxycycline, during the COVID-19 pandemic in both the United Kingdom and United States (de Lusignan et al., 2021).

In this randomized, double-blind, placebo-controlled study, we compared doxycycline and zinc with only doxycycline or placebo as prophylaxis for COVID-19 in Tunisian HCWs.

In this trial, however, the results are underpowered (69.2%) because we were unable to meet the scheduled sample size, which was estimated at a total of 1100 samples. Moreover, the recruitment of HCWs remains difficult because the enrollment of patients overlapped with the vaccination campaign. The study recruited only 15.6% of the estimated sample size.

The prophylactic effect observed with doxycycline may be attributed to the fact that it inhibits MMPs. In fact, it has been

Table 4
Patient baseline profile according to SARS-CoV-2 infection.

End point	Infected patients with SARS-coV-2	Noninfected patients	*P*-value
N	24	148	-
Diabetes	1 (4.2%)	3 (2.0%)	0.518
HTN	1 (4.2%)	6 (4.0%)	0.999
Asthma	1 (4.2%)	1 (0.68%)	0.520
COPD	1 (4.2%)	0 (0.0%)	0.279
Conveyance Own car	8 (33.34%)	60 (40.55%)	
Public transport	16 (66.67%)	88(75.68%)	0.502

Values are presented as number (percentage).
COPD, chronic obstructive pulmonary disease; HTN, hypertension.

Table 5
Safety profile of the study arms.

Adverse event	Combined therapy	Single therapy	Control group	Overall	*P*-value
N	59	56	57	172	-
Epigastralgia	7 (11.9%)	5 (8.9%)	2 (3.5%)	14 (8.1%)	0.249
Nausea	1 (1.7%)	6 (10.7%)	1 (1.8%)	8 (4.7%)	0.032
Overall	8 (13.6%)	11 (19.6%)	3 (5.3%)	22 (12.8%)	0.071

Values are presented as number (percentage).

demonstrated that coronaviruses exploit MMPs for a range of activities (replication, cell infection, and survival). Therefore, doxycycline may have an antiviral effect on SARS-CoV-2 (Dutta and Basu, 2011).

Researchers have suggested that doxycycline may delay COVID-19 progression through anti-inflammatory activities, including viruses that regulate the NF- κB pathway (nuclear factor kappa-light-chain-enhancer of activated B cells) and inhibit proinflammatory cytokine levels (interleukin 6 (IL-6), interleukin 1β (IL-1β), TNFα tumor necrosis factors (TNFα)) during acute respiratory distress syndrome (ARDS) in severely ill patients with COVID-19 (Anwar et al., 2020). Earlier studies demonstrated the effectiveness of chemically modified tetracyclines against SARS, preventing septic shock and ARDS development (Griffin et al., 2010). Additionally, several case reports suggest beneficial effects of doxycycline preventive treatment. In this context, a study demonstrated an improvement of clinical symptoms after treatment with standard doses of doxycycline in 4 patients with COVID-19 with a high-risk pulmonary disease (Yates et al., 2020).

Additionally, the combination of doxycycline and zinc also seems interesting because doxycycline has been shown to protect against lung infection by inhibiting MMPs, in which it is dependent on zinc (Doroszko et al., 2010). An interesting result of this clinical trial is the reduction in viral load in the combined group therapy (doxycycline and zinc) compared with other groups. This finding is supported by cumulative Ct values at day 49, which shows an inverse relationship with viral messenger ribonucleic acid (Supplementary Figure 1). These results are in agreement with other studies, which have suggested that Ct values are inversely associated with the viral load, and every threefold increase in Ct values indicates a 10-fold decrease in starting material (Tom and Mina, 2020). Low Ct values were also reported to be associated with virus growth in cell culture and infection (Platten et al., 2021). It is possible that the antiviral effect of zinc and inhibiting MMPs with doxycycline may help repair the damaged lung tissue, thus decreasing the virus' availability in the nasal tract and enhancing recovery.

The potential effect of combined therapy may be explained by the ability of doxycycline to catalyze Zn^{2+} ions, which are required for the activity of MMPs, independently of its antimicrobial properties (Castro et al., 2011). Doxycycline is the most potent tetracycline derivative inhibitor of MMPs, even at low doses (25 mg) (Castro et al., 2011). It may act as an ionophore by increasing intracellular zinc concentrations, suppressing viral replication, and strengthening the immune system (Griffin et al., 2010; te Velthuis et al., 2010). In addition to doxycycline's effects, zinc also has a beneficial role in protecting against COVID-19 infection. In fact, many reports have shown that zinc can inhibit the enzymatic activity and replication of SARS-CoV-2's ribonucleic acid polymerase and can inhibit angiotensin-converting enzyme activity (Ratia et al., 2006; Skalny et al., 2020). It was suggested that zinc can prevent fusion with the host membrane, decrease the viral polymerase function, impair protein translation and processing, block viral particle release, and destabilize the viral envelope (Wessels et al., 2020). Therefore, zinc has anti-inflammatory and antioxidative properties and underlying mechanisms, which have been the focus of numerous studies (Wessels et al., 2017).

In this study, we recruited 193 HCWs for 4 months (from November 2020 to February 2021). The percentage of HCWs who tested positive for COVID-19 was 21 (24%). A meta-analysis conducted during the first 6 months of the pandemic from January 1 to July 9, 2020 found a percentage of infected HCWs of 51.7% (Gholami et al., 2021). These results show that HCWs are at high risk for COVID-19 infection; thus, prevention strategies should be developed in this category. This will have a positive impact on patient's safety and could decrease the absenteeism level.

Our study was stopped after 4 months due to the start of the vaccination campaign, which prioritized HCWs (Kefi et al., 2021). We achieved 15.6% of the estimated sample size. Therefore, we recommend additional research on chemoprophylaxis that could enhance COVID-19 vaccine and litigation strategies to protect against COVID-19.

Although diabetes, hypertension, and asthma were considered COVID-19 risk factors (Gómez-Ochoa et al., 2021), they were not significantly associated with an increased risk of infection in the sample studied. Moreover, we did not consider the adherence of the HCWs to protective measures. The risk of COVID-19 infection directly depends on our physical preventive measures.

Despite the low number of participants in this study, the data suggest that a combined treatment of doxycycline and zinc may have a potential preventive effect. Public health suggestions can be made on the basis of this information for a wider adaptation of prophylaxis treatment in HCWs and the general population. Additional studies should be conducted considering vaccination laws.

FOI 26-3132 - Document 5

Conclusion

The only indication approved by the US Food and Drug Administration for the use of doxycycline in patients is as a malaria prophylaxis (<4 months). According to the portal *clinicaltrials.gov*, to date, there are not less than 405 clinical trials registered involving doxycycline, targeting different diseases, such as bacterial infections, cystic fibrosis, acne, vascular diseases, and HIV-AIDS. However, the prescription of long-term antibiotics must be cautious and supervised. Overuse is linked to bacterial resistance and alterations in gut microbiota, which have been related to risks of various chronic diseases. In this study, doxycycline combined or not with zinc has been used before the beginning of vaccination campaign. Nevertheless, this alternative therapy might be useful for certain HCWs and/or others who have comorbidities and are not responding to vaccines or have refused them.

Taking preventive measures decreased the risk of contamination by SARS-CoV-2. In this study, doxycycline and zinc were found to have a protective effect when taken together. Chemoprophylaxis can be used in conjunction with COVID-19 vaccination and personal protective measures, but more research is needed.

Funding

This trial was conducted with funds from the Military Hospital of Tunis.

Ethical statement

This study has been approved by the local ethics committee of the General Directorate of Military Health.

Author contributions

NS, HG, RB, MBM, MAY: protocol redaction; NS, RB, MBM, HG: conceptualization; NS, RB, MBM, HG: methodology; NS, KT, RB, HG: formal analysis; NS, KB, AD, RB, GH, RR: writing; SN, AD, BA, KB, RB, HG: review and editing; AB, RA, SH, SB, AR, NI, MG, AH, CA, AH, FG: investigation; MAY: product manager, supervision; MBM: virological testing; RR, KT: validation, resources, software, visualization, statistical analysis; MF, HG: project administration.

Conflict of interest statement

The authors have no competing interests to declare.

Acknowledgments

This project has been made possible owing to the efforts of Colonel Major Fethi Mattoussi, General Director of the Military Hospital of Tunis, and General Jalel Hmida, Director of the Care Services.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.06.016.

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Research Square

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Rapid increase of SpO2 on room air for 34 severe COVID-19 patients after ivermectin-based combination treatment: 55-62% normalization within 12-24 hours

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Research Article

Keywords: COVID-19, SARS-CoV-2, spike protein, ivermectin, doxycycline, SpO2

Posted Date: November 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1048271/v1

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Abstract

Background. The emergence of COVID-19 in March 2020 challenged Zimbabwe to mount a response with limited medical facilities and therapeutic options. Ivermectin (IVM) had by then been safely used to treat a variety of human diseases affecting millions, as noted by the Nobel Committee in awarding its 2015 prize for medicine. Based upon early clinical indications of efficacy against COVID-19, IVM-based combination treatments were deployed to treat this infection in Zimbabwe.

Methods. Data were retrospectively analyzed for 34 severe COVID-19 patients treated with IVM-based combination therapy between August 2020 and May 2021, for whom pre- and post-treatment SpO2 values were all recorded on room air. Mortality and deterioration outcomes were also analyzed for a larger set of 92 severe COVID-19 patients receiving IVM-based treatment.

Results: For the 34-patient SpO2 tracking series, all but two patients had significantly increased SpO2 values after the first IVM dose, and all patients recovered. Mean increases in SpO2 as percentages of full normalization to SpO2=97 were 55.1% at +12 hours and 62.3% at +24 hours post-treatment. These results paralleled similar sharp increases in SpO2, all on room air, for a series of 24 RT-qPCR confirmed, mostly severe COVID-19 patients in the USA (California) who were given IVM combination treatment, all of whom recovered. For 19 of those patients having SpO2 $\leq$ 90 prior to IVM, the mean SpO2 normalization at +24 hours post-treatment was 65.2% as calculated from the SpO2 values reported. For our larger series of 92 severe COVID-19 patients in Zimbabwe, median age 53, only two died and two more deteriorated prior to recovery, far less than a predicted 7 deaths and 17 deteriorations for the demographics and risk factors of these patients.

Conclusions. The rapid, marked increases in SpO2 for both the Zimbabwe and California patients stand in sharp contrast to the decline in SpO2 and associated pulmonary function following onset of moderate or severe COVID-19 symptoms under standard care. These rapid SpO2 increases and low mortality rates support extended deployment of IVM treatment for COVID-19, complementary to immunizations for prevention.

Background

Evaluations of efficacy for COVID-19 therapeutics are challenging since most patients with this disease typically recover; the worldwide cumulative case fatality rate is 2% as of September 2021.[1]. Thus, randomized clinical trial (RCT) results are important, yet interpretations, for example, of the more than 20 mostly positive RCT results for COVID-19 treatment with the drug ivermectin (IVM), a macrocyclic lactone used widely worldwide since 1987, as summarized below, are controversial. Even for a drug such as remdesivir that was tested with several RCTs having large patient cohorts, opposite conclusions were drawn by the US FDA[2] and the World Health Organization[3] as to efficacy against COVID-19.

As a complement to positive RCT results for a COVID-19 therapeutic, a quantifiable determination of rapid, major improvement in pulmonary function, especially for severe COVID-19 patients, would further indicate efficacy and provide insights into that drug's underlying biological mechanism. The simplest indicator of lung function is blood oxygen saturation level, SpO2, as detectable using a pulse oximeter. However, the immediate administration of supplemental oxygen to any severe COVID-19 patient would typically preclude meaningful

pre-and post-treatment comparisons of SpO2 values. For this retrospective study conducted in Zimbabwe, ironically, challenges that constrained treatment capabilities also provided the opportunity to track changes in SpO2, all on room air, for 34 severe COVID-19 patients within 12 to 48 hours after beginning IVM treatment (see Figure 1). At the same time, these challenges tested whether severe COVID-19 patients with limited opportunities for hospitalization could be successfully treated at home or in clinics with rudimentary facilities.

Zimbabwe, a landlocked country in Southern Africa that shares a border with South Africa, had its first reported case of COVID-19 in March 2020.[4] Eight cases and one death from COVID-19 followed in the same month.[5] The first death occurred in the Wilkins hospital, Zimbabwe's main COVID-19 treatment center in the capital city of Harare. Facilities were limited at this hospital at the time; no capacity for ventilation was available. Given the rapid increase in COVID-19 patients in Harare after March 2020,[6] additional medical facilities began treating them.

COVID-19 wards were created at a general practice clinic by converting two staff rooms into a 4-bed ward and a storeroom into a 2-bed ward. Available equipment included several oxygen cylinders, an oxygen concentrator, six beds, and three monitors for SpO2 and blood flow parameters. The staff consisted of the lead author and another primary care physician who was off duty for several weeks after being injured in a vehicle accident on July 27, 2020, and either one or two nurses at different times, each on 12-hours shifts. During the initial months of the pandemic, in the absence of proven therapies and protocols, the standard of care evolved through early August 2020 to include corticosteroids, clopidogrel, aspirin, enoxaparin, rivaroxaban, a nebulized nano-silver preparation, zinc sulfate, hydroxychloroquine, azithromycin, doxycycline and in some cases an IV antibiotic.

However, the efficacy of these treatments was found to be limited, and by the end of July 2020, several COVID-19 deaths were recorded in the country. Based upon reports of initial success using IVM for COVID-19 treatment from colleagues in Johannesburg, South Africa, the College of Primary Care Physicians of Zimbabwe (CPCPZ) adopted and included IVM in their COVID-19 treatment protocol from August 8, 2020, starting initially with a 10-12 mg stat dose. Treatment of COVID-19 with IVM continued after the first patients showed improved outcomes, with more rapid recoveries achieved at doses higher than the standard of 200 ug/kg as initially used.

In August 2020, after it became apparent that IVM added to standard of care was significantly reducing the death rate, together with the hospital system being overwhelmed, CPCPZ physicians decided to treat COVID-19 patients where an IVM-based protocol could be administered, including at local general practice clinics which had nursing care and oxygen, and at some patients' homes with nursing support and oxygen supplementation as available. As knowledge of this successful treatment regimen spread in Zimbabwe, other physicians began offering the same treatment, with improved outcomes, which led to the formation of the Zimbabwe COVID Front Line Clinicians Society.

IVM for COVID-19 treatment

The decision to include IVM in COVID-19 treatment protocols in Zimbabwe was made as the pandemic swept through that nation, overwhelming limited clinical care facilities, with no drug developed to treat COVID-19

being generally accepted as effective. A published case-controlled study of IVM treatment for COVID-19 conducted at four US hospitals[7] that had been initially released in a preprint in June 2020 found a 40% reduction in mortality among 173 patients treated with low dose IVM vs 107 case-matched controls (15% vs 25.2% deaths). Interest in IVM was supported by its Nobel prize-honored pedigree and its extensive use to treat a variety of human diseases in over 3.7 billion doses worldwide since 1987.[8–10] Another favorable characteristic of this drug is its extraordinary record of safety, well tolerated at high doses,[11,12] including in studies for COVID-19 treatment.[13,14] It is generally non-toxic even at doses far exceeding the therapeutic range.[15,16] Since August 2020, inpatient and outpatient treatments of COVID-19 with IVM have been applied across 25 countries,[10] with more than 20 RCTs conducted for IVM treatment regimens.[10,17,18]

Seven of nine meta-analyses of these RCTs for IVM treatment reporting in 2021, all conducted using Cochrane analysis methodology, found significant[18–22] or possible[23,24] indications of IVM efficacy, with a mean 0.33 relative risk (RR) of mortality vs controls. Most of these 20 RCTs for IVM treatment of COVID-19 showed statistically significant mortality reductions or other clinical benefits. Among the most recent and detailed of the nine meta-analyses noted above reported a pooled total 67% reduction in mortality for IVM vs controls, with a statistical significance for an overall effect of p=0.005.[20] A comprehensive review of the entire body of clinical studies for IVM treatment of COVID-19 by the Nobel co-laureate for IVM, Dr Satoshi Omura and colleagues, concluded that IVM yielded major reductions in mortality.[10] Two animal studies of IVM treatment at low human-equivalent doses, one for the SARS-CoV-2 virus in golden hamsters[25] and another for a related betacoronavirus (MHV-A59) in mice,[26] found statistically significant treatment benefits, consistent with those found in the RCTs noted above. The indicated biological mechanism of IVM, competitive binding with SARS-CoV-2 spike protein,[27] is likely non-epitope specific, possibly yielding full efficacy against emerging viral mutant strains.

The demonstrated safety of IVM at much higher than standard doses[11–14] allowed the latitude for dose escalation for IVM treatment of COVID-19 over time. On September 19, 2020, the CPCPZ held a seminar at which the use of a combination of IVM, doxycycline and zinc was presented, along with aggressive diabetes control, steroid use and anticoagulation, and this was suggested as the most effective and affordable care available at the time. Afterwards, combination therapy centered around IVM plus doxycycline and zinc became the standard COVID-19 treatment protocol used by the CPCPZ. The potential efficacy of these adjuncts was later supported by successful clinical trials results with treatments using IVM in combination with doxycycline[28] or with doxycycline and zinc.[29] This combination therapy for COVID-19 has been researched and advanced by Thomas Borody,[30] who in 1990 published the first clinical trial of using a triple therapy of three inexpensive repurposed drugs for *H. pylori*,[31] the underlying bacterial cause of peptic ulcers. This triple therapy of repurposed drugs became the worldwide standard of care for peptic ulcers a decade later, after the patents for the palliative drugs Tagamet and Zantac expired, and the discovery of *H. pylori* as the cause of peptic ulcers was honored with the Nobel Prize for Medicine in 2005.[30]

Methods

This study is a retrospective review of clinical data collected during the course of treatment of COVID-19 patients with therapeutic agents selected by their physicians to offer the greatest chances for clinical benefits and recovery.

Outcomes

Outcomes tracked were 1) changes in SpO2 values from within one hour before treatment to 12, 24 and 48 hours after treatment, for a set of 34 COVID-19 patients for whom a pre-treatment SpO2 value and at least one SpO2 value up to +48 hours after start of treatment (first IVM dose) were available, all obtained on room air; and 2) mortality/deterioration: recovery, deterioration to critical status, or death for a larger set of 92 COVID-19 patients. For most of these patients, blood values for lymphocyte count, LDH, D-Dimer and CRP were also recorded, but complete blood test results were not obtained for every patient and therefore were not analyzed.

Participants

Sixty of the patients analyzed in this study, including all 34 in the SpO2 tracking series, were from Harare, treated by CPCPZ physicians either at local clinics or at patients' homes. Additional data for the mortality/deterioration series were obtained through inquiries sent on March 10, 2021, to all in a WhatsApp group of CPCPZ physicians treating COVID-19 with IVM asking for records of any of their patients so treated. Six physicians responded by furnishing records of 32 such patients, grouped with the 60 patients treated by CPCPZ physicians to comprise the mortality/deterioration series. For most of these six physicians, not every patient treated to recovery without incident was included, but it was confirmed that no other patient treated by them with IVM had died. Under pressures of patient care during the pandemic, record-keeping tended to be most comprehensive for the sickest patients, who thus are represented disproportionately in this study.

Table 1
Age group, sex, and pre-treatment SpO2 (%) value range of the 92-patient mortality/ deterioration series (mean age=54; median age=53).

Age		Initial SpO2 (%)			
Group	**Sex**	**66-84**	**85-89**	**90**	**Total**
25-49					
	Female	5	9	1	15
	Male	6	11	0	17
	Total	**11**	**20**	**1**	**32**
50-59					
	Female	2	6	0	8
	Male	8	8	2	18
	Total	**10**	**14**	**2**	**26**
60-69					
	Female	4	6	0	10
	Male	6	7	0	13
	Total	**10**	**13**	**0**	**23**
70-79					
	Female	1	1	1	3
	Male	1	0	2	3
	Total	**2**	**1**	**3**	**6**
80+					
	Female	0	2	0	2
	Male	1	2	0	3
	Total	**1**	**4**	**0**	**5**
TOTAL		**34**	**52**	**6**	**92**

Inclusion and Exclusion criteria

In both the SpO2 tracking and mortality/deterioration series, patients selected for analysis were of age 18 or older and had treatment start dates between August 8, 2020, and May 31, 2021. Patients selected had an SpO2 value on intake of 51% or above and were administered a treatment protocol including IVM. Furthermore, patients selected were required to have been found COVID-19 positive either by a PCR test or a clinical diagnosis made by criteria including exposure to a COVID-19 patient, hypoxia, lymphopenia,

monocytosis, elevated LDH, elevated dimer, and/or radiology consistent with pulmonary abnormalities caused by the virus.

For the mortality/deterioration analysis, the set of patients was further restricted to those with pre-treatment SpO2 of 90% or below (and a minimum SpO2 for all patients of 51%, as noted above). Table 1 shows the distribution of age, sex and pre-treatment SpO2 value of the 92 patients in this series. For the SpO2 tracking series, the subset of patients was restricted to those patients with pre-treatment SpO2 values of 51% through 93%. That series was further restricted to those for whom these SpO2 values were recorded and positively documented to have all been obtained on room air (in almost every case because oxygen was not available), and at least one SpO2 value obtained within 48 hours after IVM administration, with 34 patients fitting those criteria. Six of those patients had pre-treatment SpO2 values of 91-93% and were thus not included in the 92-patient mortality/deterioration series. All patients in both the mortality/deterioration and SpO2 tracking series thus fit the US National Institutes of Health's definition of severe COVID-19, a sufficient condition of which is SpO2 of 93% or below.[32]

Treatment

In the 92-patient mortality/deterioration series and in the overlapping 34-patient SpO2 tracking series, every patient received IVM at dosages described below in addition to selected other agents from the standard of care before August 2020. These other agents included corticosteroids, clopidogrel, aspirin, enoxaparin, a nebulized nano-silver preparation, rivaroxaban, zinc sulfate, azithromycin, doxycycline, and in some cases, an IV antibiotic. Patients treated in a clinic were assessed by a nurse upon admittance, with blood drawn and PCR tests conducted as feasible given the patient's condition and with severe symptoms necessitating immediate treatment. For those patients who contacted a CPCPZ physician from home requesting treatment, an online questionnaire was first completed by the patient, after which, if COVID-19 was still a suspected diagnosis, a nurse visit to the home was conducted, with associated follow up per the procedures described for in-clinic patients.

As evidence of IVM safety and tolerability accrued following its use beginning in August 2020, its stat dose of 10 mg as used for the earliest patients was increased on September 11, 2020, to 10-12 mg every four days for three doses. Subsequently, the dosage was further increased to 12 mg IVM on the day of admission and then on days 4 and 8 plus doxycycline (100mg b.i.d.) and zinc sulfate (60mg/day). The latter regimen was used up through December 2020, when the second pandemic wave emerged in Zimbabwe. At that time, additional evidence of safety and tolerability of this regimen supported further dose escalation to a standard IVM dose regimen of 12 mg daily for five consecutive days, with adjunct use of doxycycline and zinc sulfate continued at the doses noted. In some cases, for which this standard treatment regimen did not yield significant clinical gains within a few days, even higher doses of IVM were used, in some cases as high as 100 mg for a single dose. Transient adverse effects (AEs) such as blurred vision characteristic of high dose IVM often occurred at those dose levels, but no serious AEs associated with IVM were manifested in any patient. Each of the 34 patients in the SpO2 tracking series was treated with IVM, doxycycline and zinc.

Data collection

For patients in the SpO2 tracking series that were treated in clinics, values were tracked using monitors that continually displayed SpO2 values and readings for pulse rate and blood pressure and waveform images of blood pulses. For those treated at home, the intake nurse provided a pulse oximeter to the patient, unless the patient had one which the nurse deemed of reliable quality. The patient or a family member took SpO2 readings regularly, using the same oximeter as used for the pre-treatment reading. In most cases, these readings were taken daily, with much lower frequency after SpO2 values had risen significantly and the patient's clinical condition had correspondingly improved. Patients were instructed to message the nurse immediately if SpO2 ever decreased from a higher value to 93% or below or if any new clinical symptoms of concern developed.

Patient outcomes were categorized as recovered, died, or deteriorated before recovery, as follows. Recovered: patients who recovered following IVM treatment and were still alive on September 1, 2021, three months or more after intake. Died: those who died following IVM treatment, whether or not the cause could have been unrelated to COVID-19. Deteriorated (before recovery): if at any time following the first dose of IVM, any incident or condition, whether of indicated connection to COVID-19 or not, either caused them to be hospitalized or made that a reasonable course of action had that been an available and viable option.

Analytical methods

For the 34-patient SpO2 tracking series, a measure of percent normalization toward a fully optimal SpO2 value of 97 was applied, which for pre-and post-treatment SpO2 values S_0 and S_1, is: $100*(S_1-S_0)/(97-S_0)$, capped at 100%. Pre- and post-treatment SpO2 values were plotted for all 34 patients using this percent normalization measure in Figure 1 and were also presented directly in Figure 2, Figure 3, and Table S2. Regarding the 92-patient mortality/deterioration series, precise statistical comparisons of results from treatment vs control groups cannot be made other than in the context of an RCT. It is nevertheless of interest to compare the mortality results for this Zimbabwe mortality/deterioration patient series to the expected mortality of COVID-19 patients with similar characteristics. A review conducted in 2021 considered 46 prediction models for COVID-19 mortality,[33] two of which[34,35] were rated as having a low risk of bias; the same two were identified as being of the highest quality in another overview of COVID-19 mortality risk assessment models.[36]

The risk assessment model used here is the one of these two that used pre-treatment SpO2 as one of its prediction variables.[35] This model, designated as the 4C mortality risk predictor, was developed by 35 investigators on behalf of a consortium of 260 hospitals in the UK. It was derived from 57,824 hospitalized patients, a comparable group to this study's mortality/deterioration series of patients, who, as noted, all had pre-treatment SpO2 values ≤ 90% and would have been hospitalized had that been an available, viable option. The overall mortality rate of 4C model patients, using data collected February through June 2020, was 31%. In Harare's main hospital system, the Parirenyatwa Group, in its red zone, where COVID-19 patients are admitted and treated, per statistics available for June through December 2020, the COVID-19 case fatality rate (CFR) was 35.4% (119 deaths of 336 total patients).[37] This exceeds the overall CFR for the patient set of the 4C mortality risk predictor and indicates that using the 4C mortality risk predictor for the patient set in this study would not overestimate the risk factor.

The same UK consortium that developed the 4C mortality predictor also developed a model to assess the risk of clinical deterioration among inpatients with confirmed or highly suspected cases of COVID-19.[38] This deterioration model was developed using 73,948 patients recruited between February and August 2020, with clinical deterioration defined as any requirement of ventilatory support or critical care, or death. This model was also applied to the Zimbabwe mortality/deterioration series, and predicted deterioration outcomes were compared with the number of patients who could be considered to have deteriorated, per the criteria specified above.

To calculate both mortality and deterioration probability estimates for the Zimbabwe mortality/deterioration series, three base variables were used: age, sex and pre-treatment SpO2 value on room air, while for mortality, the 4C model used the count of comorbidities as another variable, as was also recorded for the study patients. Abnormal values for respiratory rate, urea, CRP, lymphocyte count, and presence of radiographic chest infiltrates would have added extra points and would have increased associated probabilities to these risks calculated for mortality and/or deterioration. However, since only these four base variables were available for every patient, only these were used to calculate the 4C-predicted risks.

The comorbidities used in the 4C mortality predictor were the following: chronic cardiac disease, chronic respiratory disease (excluding asthma), chronic cardiac disease, chronic respiratory disease (excluding asthma), chronic renal disease, mild to severe liver disease, dementia, chronic neurological disease, connective tissue disease, diabetes, HIV or AIDS, malignancy, and clinician-defined obesity.[38] For applying this risk predictor to the Zimbabwe mortality/deterioration patient series, these were the comorbidities used for the counts shown in Table S1. For some of these patients, certain variables used for the deterioration but not the mortality risk calculation would likely have significantly increased the deterioration probability values, and the calculated deterioration risk estimate is lower than that for mortality.

The 4C mortality and deterioration risk predictors were used to calculate probabilities for each of the 92 patients for mortality and deterioration, respectively. Although the Poisson distribution function can roughly approximate the probability of having a given number n or less total events from such a series having different probabilities, Monte Carlo simulation gives a much more precise probability estimate.[39] Monte Carlo simulations were executed, with 10,000,000 simulations performed ten times each for the mortality and deterioration estimations. These were performed using visual basic source code as listed in Supplementary File S3.

Ethics Approval

The Medical Research Council of Zimbabwe granted IRB approval (#E293) for this retrospective study. Patients consented for their medical treatment and to having their de-identified data used in this study.

Results

For COVID-19 patients treated with IVM in Zimbabwe between August 8, 2020, and May 31, 2021, results are presented for the following: A) Pre- and post-treatment SpO2 values for 34 patients for whom these values were recorded all on room air, each patient having a pre-treatment SpO2 value of 51-93% and at least one

post-treatment value obtained within 48 hours after first dose of IVM; and B) analysis of mortality and deterioration outcomes for 92 patients having pre-treatment SpO2 values of 51-90%.

Results for pre-and post-treatment SpO2 values

Figures 1 and 2 and Table S2 show the progression of pre-treatment and post-treatment SpO2 values within 48 hours after first dose of IVM for the 34 patients described above, all of whom were treated with IVM, doxycycline and zinc. All of these patients recovered. SpO2 values are shown at pre-treatment, all recorded within one hour before the start of treatment and at 12, 24 and 48 hours after treatment. The SpO2 value shown for a given patient at time x is that for the latest post-treatment time $\leq$ x. (Thus, for some patients, for example, having a post-treatment value within 12 hours before +24 hours but none in the next 24-hour period, the latest value at +48 hours is the same as that at +24 hours.)

Figure 1 shows, for all 34 patients, SpO2 value changes from before IVM administration to post-treatment as percentages of full normalization to an optimal SpO2 value of 97 (95 is considered the minimum normal SpO2 value for a healthy child or adult by the US CDC[41]). Red, orange and blue lines show, respectively, SpO2 values at +12, +24 and +48 hours post-treatment. The mean (±SD) SpO2 changes as this specified percent of optimal normalization were 55.1% ± 28.0% at +12 hours, 62.3% ± 26.3% at +24 hours and 64.3% ± 24.5% at +48 hours. As shown in Figure 1, these percentages of full normalization to SpO2=97 have a roughly uniform distribution across the full range of pre-treatment SpO2 values, from 66 to 93.

Figure 2 shows all pre-and post-treatment SpO2 values grouped into nine graphs (A-I) by the range of pre-treatment SpO2 values and post-treatment times (after first IVM dose) of +12, +24 and +48 hours. As shown in Figure 2 and identically in Table 2, those patients with lowest, mid-range and highest pre-treatment SpO2 values had mean SpO2 increases at +12 hours of 12.8, 5.4 and 2.8, respectively (all SpO2 values in percentage units). Figure 3 shows pre-and post-treatment SpO2 values throughout the entire observation period for each of the 34 patients. Note that these values were recorded less frequently after SpO2 had normalized.

Table 2
Mean ± SD of changes in SpO2 from pre- to post-treatment (after first IVM dose) for 34 severe COVID-19 patients treated with IVM, doxycycline and zinc.

SpO2 (%)			
Pre-treatment	at +12 hours	at +24 hours	at +48 hours
66 - 84%	+12.8 ± 6.7	+11.4 ± 6.1	+11.7 ± 5.8
85 - 89%	+5.4 ± 2.6	+6.2 ± 2.8	+6.3 ± 3.0
90 - 93%	+2.8 ± 2.7	+3.7 ± 2.3	+3.9 ± 1.8

Figure 4 shows successive SpO2 values for one patient who had a particularly rapid increase in these values after his first dose of IVM. This patient was a 25-year-old male, treated by a CPCPZ physician at a GP clinic without a supplemental oxygen capability. He received his first 12 mg IVM dose (repeated over the next four

days) immediately after entering the clinic with respiratory distress and bilateral pneumonia indicated by stethoscopic examination. His COVID-19 diagnosis was confirmed by a positive result from a rapid antigen test. As shown, his SpO2 values increased from that recorded immediately before treatment (79%) to values at 45 minutes (87%), 90 minutes (92%) and 3 hours (95%) post-treatment. He was discharged later that same day, and his home SpO2 readings then fluctuated between 92% and 95% over the next three days. By the fourth day after discharge, his SpO2 stabilized at 97%, his pulse dropped to 77 from prior values over 100, and he resumed working from home.

For the 34 patients in the SpO2 tracking series, as shown in Figure 2, all but two had increases in SpO2 within the first 48 hours after first dose of IVM. As shown in Figure 3, this overall increase in SpO2 continued throughout the entire observation period. However, several studies of moderate and severe COVID-19 patients under standard care that track SpO2, pulmonary abnormalities, or both establish that for most patients, SpO2 decreases in tandem with an increase in the extent of pulmonary CT abnormalities from the day of onset of disease symptoms through the second week following.[42–48] Thus, the expected change in SpO2 within 48 hours after first IVM dose would be < 0, and one-tailed paired t-test calculations can assess whether SpO2 values increased significantly > 0 at each time period tracked.

Taking into account some missing post-treatment values (see Table S2), there are 25, 33 and 34 pairs of pre- and post-treatment SpO2 values at +12 hours, +24 and +48 hours, respectively. Applying these paired t-test calculations, the SpO2 increases were highly significant for each time period: t=6.28, p=8.5E-07 at +12 hours; t=8.42, p=6.36E-10 at +24 hours, and t=8.81, p=3.47E-10 at +48 hours.

Results for mortality and deterioration

The mortality/deterioration series, as noted above, consisted of 92 COVID-19 patients with pre-treatment SpO2 values between 51% and 90%. Table S1 shows individual values for these patients for age, sex, pre-treatment SpO2 value, number of comorbidities, treatment outcome (recovered, deteriorated or died), and the respective probabilities from the 4C mortality and deterioration risk predictors.

Of these 92 patients, 90 recovered, and two died. (The latter two patients did not have recorded SpO2 values on room air and were not included in the 34-patient SpO2 tracking series.) To compare this result with the mortality outcome expected for a series of 92 COVID-19 patients with matching values of age, sex, pre-treatment SpO2 and the number of comorbidities, 4C mortality probabilities were calculated for each patient. Monte Carlo simulations were then executed, with 10,000,000 simulations run ten times. The number of simulated deaths in each run ranged from 7.079 to 7.081, with a mean of 7.080, and the mean of the associated individual standard deviations = 2.494. The probability of having zero to two simulated deaths in this series ranged in these ten runs was 0.0214 (identically for mean, minimum and maximum values).

Two patients other than the two who died fit the deterioration criteria noted above. One was transferred to a hospital for three days because he became weak from not eating, and another was hospitalized for six days due to a drop in oxygen saturation. Both resumed IVM treatment after returning home and subsequently recovered. These two plus the two patient deaths yield a total of four deteriorations according to the 4C risk model for deterioration. 4C deterioration probabilities were calculated for each of the 92 patients, and Monte Carlo simulations were then executed for deterioration risk estimation, with 10,000,000 simulations run ten

times. The number of simulated deteriorations in each run was 17.23 (identically for the mean, minimum and maximum of these values), with the mean of the associated individual standard deviations = 3.54. The probability of having zero to four simulated deteriorations in this series ranged in these ten runs from 0.0000196 to 0.0000216, with a mean of 0.0000207.

With the caveat above as to the limits of statistical analyses outside the context of an RCT, this calculation nevertheless suggests that the occurrence of four or fewer deteriorations among these 92 severe COVID-19 patients would be highly improbable under standard care. For comparison, note that in this series of 92 patients, 21 had pre-treatment SpO2 values ≤ 78%, and among these 21 patients, only two deteriorated (the patients who died). In sharp contrast were deterioration outcomes reported by Mukhtar et al. in a study of 72 critically ill COVID-19 patients.[49] Thirty-four of those 72 patients had hospital admission SpO2 values ≤ 78% on room air, and all of those subsequently required mechanical ventilation, whereas only 16 of 38 (42%) of patients having intake SpO2 value > 78% required ventilation (Mohamed Hasanin, corresponding author of this study, personal communication, August 15, 2021).

Discussion

This study is a retrospective review of clinical data collected amid the challenges of providing treatment with limited facilities and resources to COVID-19 patients with severe disease. Under such conditions, it was not possible to obtain blood test values for all patients, including values for lymphocyte count, LDH, D-Dimer and CRP, which were thus not analyzed. On the other hand, the lack of availability of oxygen supplementation for many of the patients treated resulted in the rare opportunity to track changes in SpO2 values all recorded on room air before and after administration of IVM to 34 patients, with several of these patients having presented with SpO2 values well below 90%.

The increase in SpO2 for these 34 patients as the percentage of fully optimal normalization to SpO2=97, as reported above, was (mean ±SD) 55.1% ± 28.0% at +12 hours, which rose to 62.3% ± 26.3% at +24 hours and then to 64.3% ± 24.5% at +48 hours after first IVM dose. All but two of these 34 patients had SpO2 values that increased at all post-treatment times for which values were obtained. Paired t-test calculations yield $p<0.0000001$ for the SpO2 increases at +12, +24 or +48 hours having occurred by chance had the IVM-based combination therapy applied had no clinical activity against COVID-19. Because the dearth of significant spontaneous improvements in SpO2 levels and respiratory function one day after a severe COVID-19 patient's presentation for medical care is a well-established norm for this disease per the studies cited above, these probability values are significant and noteworthy.

The results for this SpO2 tracking series, with recoveries for all 34 patients, parallel those recently reported by Hazan et al., for which SpO2 values all on room air for 24 RT-qPCR confirmed COVID-19 patients were tracked before and +24 hours after combination treatment with IVM, doxycycline and zinc.[40] For the 19 severe COVID-19 patients in that series who had pre-treatment SpO2 values of 90% and below (minimum=77%) and had +24 hour post-treatment SpO2 values (see Table S3), the mean (±SD) SpO2 values were 86.7 ± 4.5 pre-treatment and 93.3 ± 2.6 at +24 hours after first IVM dose. As the percentage of normalization to SpO2=97, the mean (±SD) relative SpO2 increase for these 19 patients was 65.2% ± 17.5%, which is close to the +24 hour relative increase of 62.3% for the 34-patient Zimbabwe SpO2 tracking series. The one-tailed paired t-test for these

increased SpO2 values in these 19 patients yields t=9.34, p=1.27E-08, which as for the Zimbabwe SpO2 series is highly significant.

The similar results for the SpO2 patients in the Zimbabwe SpO2 series and the 24 patients of Hazan et al. suggest that the triple therapy of IVM, doxycycline and zinc provides efficacy regardless of which other adjunct agents were used, or may indicate that each study used different sets of adjuncts that further boosted the activity of this regimen to the degree of efficacy manifested in the clinical outcomes for each. One limitation of this study is that except for nebulized nano-silver, used for all patients at the start of the treatment regimen, other adjuncts were deployed on a case-by-case basis, per the patient's condition. Also, IVM dosages were increased during the study period based on the observations that no serious AEs were observed at higher doses, and higher doses appeared to be most effective for the patients with the most severe symptoms. A follow-up clinical study of IVM-based combination treatment of COVID-19 would benefit from a more structured specification and tracking of dosages and adjuncts used.

The distinct improvements in respiratory function for this study's 34-patient SpO2 tracking series, paralleling similar results reported by Hazan et al., provide a quantifiable demonstration of rapid clinical improvement in severe COVID-19 patients after IVM treatment. Such rapid improvements had been observed since the first major clinical trial of IVM treatment of COVID-19.[7] The lead investigator of that clinical trial had observed that stabilization and then improvement in breathing function frequently occurred in 12-48 hours after IVM treatment, even for patients who had been deteriorating rapidly and had required supplemental oxygen at up to a 50% mixture.[50]

The SpO2 increases within a day after IVM treatment observed in this study and by Hazan et al. provide a distinct indication that IVM not only yields statistically significant clinical benefits in groups of patients as reported in most of the 20 RCTs for IVM treatment but provides rapid, directly observable resolution of pulmonary dysfunction as tracked by SpO2 values for COVID-19 patients. This finding offers clues as to the potential biological mechanism of IVM activity against SARS-CoV-2 since, for example, even an effective freeze on viral replication or rapid repair of damaged pulmonary alveoli would be unlikely to cause such rapid clinical improvements. One indicated biological mechanism of IVM activity, competitive binding with SARS-CoV-2 spike protein, as reviewed,[27] may, through a reversal of viral hemagglutination, act quickly to increase pulmonary capillary flow and in turn account for normalization of blood oxygenation. An additional plausible mechanism of IVM activity is the activation of the cholinergic anti-inflammatory pathway under the control of the vagus nerve,[51] which is regulated by acetylcholine and potentiated by the high-affinity binding of IVM (a positive allosteric modulator) to the alpha 7 cholinergic receptor a7nAChr[52] expressed on bronchial, vascular as well as to cytokine-producing cells (i.e., TNF, IL1 and IL6 secreting macrophages, lymphocytes and mast cells).[53]

For the SpO2 tracking series, the pattern of rapid increases in SpO2 after start of IVM treatment as occurred for all but two of these 34 patients resulted in recoveries for all of them. This same pattern of highly successful outcomes extended to the 92-patient mortality/deterioration series, with recoveries of all but two patients. As noted above, although statistical significance cannot be determined through the 4C mortality and deterioration calculations, these odds calculations suggest that the probabilities for achieving the mortality and deterioration results obtained in this study were, respectively, low and extremely low under standard care.

No serious adverse effects (AEs) from IVM treatment were observed in any patient, although transient AEs such as blurred vision characteristic of higher-dose IVM administration were observed in some patients given doses as high as 100 mg. While comparative results using higher vs lower doses were not systematically tracked, the practice of increasing IVM doses for patients not initially responding to treatment worked out well and supports the indication that higher doses provide greater efficacy.

Conclusions

Pre-and post-treatment SpO2 values were recorded all on room air for 34 severe COVID-19 patients who were treated with the combination therapy of IVM, doxycycline and zinc plus other adjuncts. This application of multiple drugs against COVID-19 was based on Zimbabwe's experience with prior infectious diseases, for which early, aggressive use of multiple drugs has been a core treatment principle. For these 34 patients, all but two had increases in SpO2 from pre- to post-treatment, at every time interval of +12, +24 and +48 hours after first IVM dose for which values were recorded. The mean increase in SpO2 value as a percentage of full normalization to SpO2=97 was 55.1% at +12 hours and 62.3% at +24 hours after first IVM dose. These results closely parallel the mean SpO2 normalization of 65.2% as calculated from SpO2 values reported by Hazan et al. for 19 RT-qPCR confirmed COVID-19 patients having pre-treatment SpO2 $\leq$ 90, with all SpO2 values on room air.

The marked, rapid normalizations of blood oxygenation, $p<0.0000001$ for the 34-patient SpO2 tracking series in each time period analyzed (paired t-test), stand in sharp contrast to the well-established typical decline in SpO2 during at least the first week after onset of moderate or severe COVID-19 symptoms and establish a cause-and-effect clinical benefit for IVM-based combination treatment of this disease. Furthermore, for the larger set of 92 severe COVID-19 patients treated with IVM and other adjunct agents, all having pre-treatment SpO2 values of 90 and below, all but two recovered, and only two of those recovering patients experienced deterioration before recovery. These two deaths and four deteriorations (including the two deaths) are much less than the expected seven deaths and 17 deteriorations predicted using the well-regarded 4C COVID-19 risk assessment model.

For the patients of this Zimbabwe study and the Hazan critical series, treatment at home or in clinics with basic facilities freed up hospital resources for other patients, and the treatment approach modeled in these studies could significantly relieve the pressure on overwhelmed health facilities. IVM is widely available worldwide, inexpensive, and one of the safest drugs in modern medicine, with its safety in "improving the health and wellbeing of millions" noted explicitly by the Nobel Committee in awarding its 2015 prize for the discovery of IVM.[54] These study results, therefore, support the extended deployment of IVM for COVID-19 treatment, complementary to immunizations for prevention.

Declarations

Funding

No funding was received for this study.

Conflict of Interests

All authors report no conflicts of interest.

Data availability statement

Data underlying the study cannot be made publicly available due to ethical concerns about patient confidentiality. Data will be made available to qualified researchers on request to the corresponding author.

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Figures

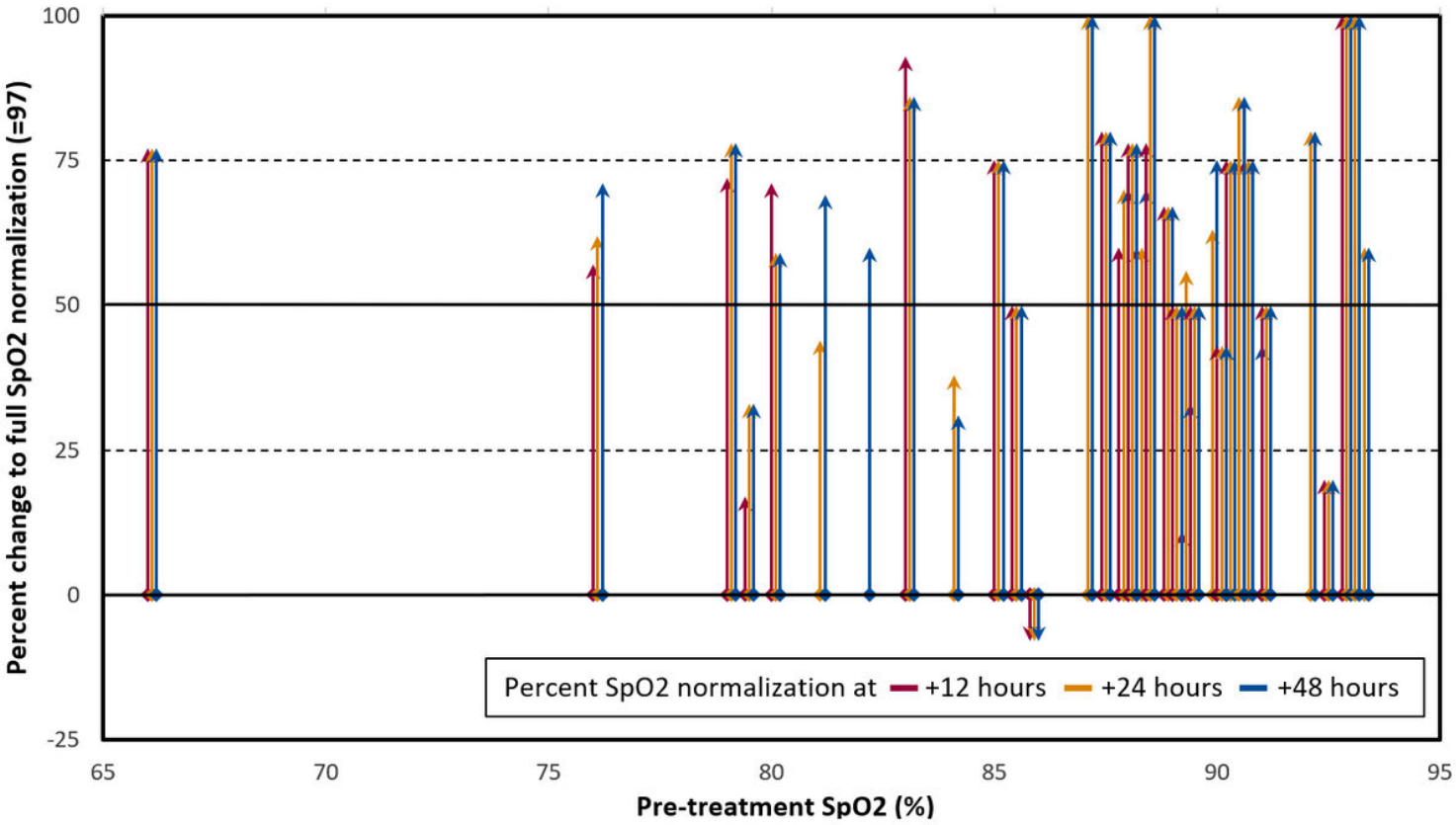


Figure 1

Changes in pre- to post-treatment SpO2 values, all on room air, for 34 severe COVID-19 patients treated with ivermectin (IVM), doxycycline and zinc, as percentages of optimal normalization to SpO2=97. Red, orange and blue lines represent, respectively, changes in SpO2 values at +12, +24 and +48 hours after first dose of IVM. X values were increased slightly (<1.5) as needed to accommodate clusters of values. For pre-and post-treatment SpO2 values S0 and S1, the y axis is the percent of optimal normalization to SpO2=97, that is: 100* (S1-S0)/(97-S0), capped at 100%. Mean (±SD) SpO2 changes (y values) were 55.1% ± 28.0% at +12 hours, 62.3% ± 26.3% at +24 hours and 64.3% ± 24.5% at +48 hours. These results closely parallel changes in SpO2, all on room air, reported by Hazan et al. for 19 RT-qPCR confirmed COVID-19 patients having pre-treatment SpO2 ≤ 90.40 At 24 hours after treatment with IVM, doxycycline and zinc, the mean SpO2 increase as a percentage of normalization to SpO2=97 was 65.2%, as calculated from the SpO2 values reported, close to the 62.3% at +24 hours for this 34-patient series. All patients in both of these IVM-treated cohorts recovered.

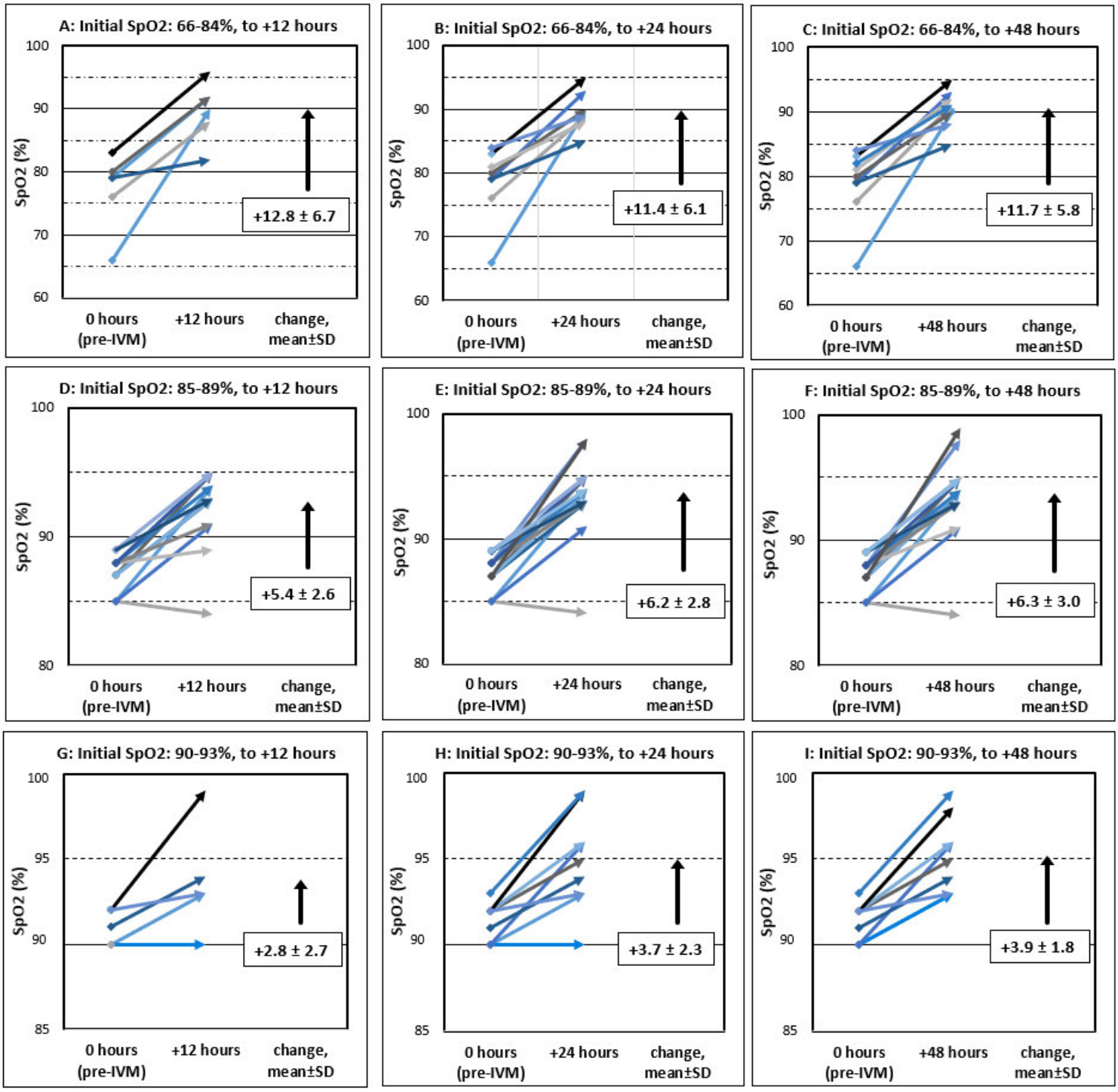


Figure 2

Pre- and post-treatment SpO2 values, all on room air, for 34 severe COVID-19 patients treated with IVM, doxycycline and zinc. Graphs A-I are in horizontal groupings by the range of pre-treatment SpO2 value and in vertical groupings by elapsed time after first dose of IVM. (The lowest SpO2 value in the stipulated range of 51-93% was 66%.) For these elapsed times of x=12, 24, and 48 hours, the SpO2 value shown for a given patient is at the latest post-treatment time ≤ x. All pre-treatment SpO2 values are from within one hour before the start of treatment.

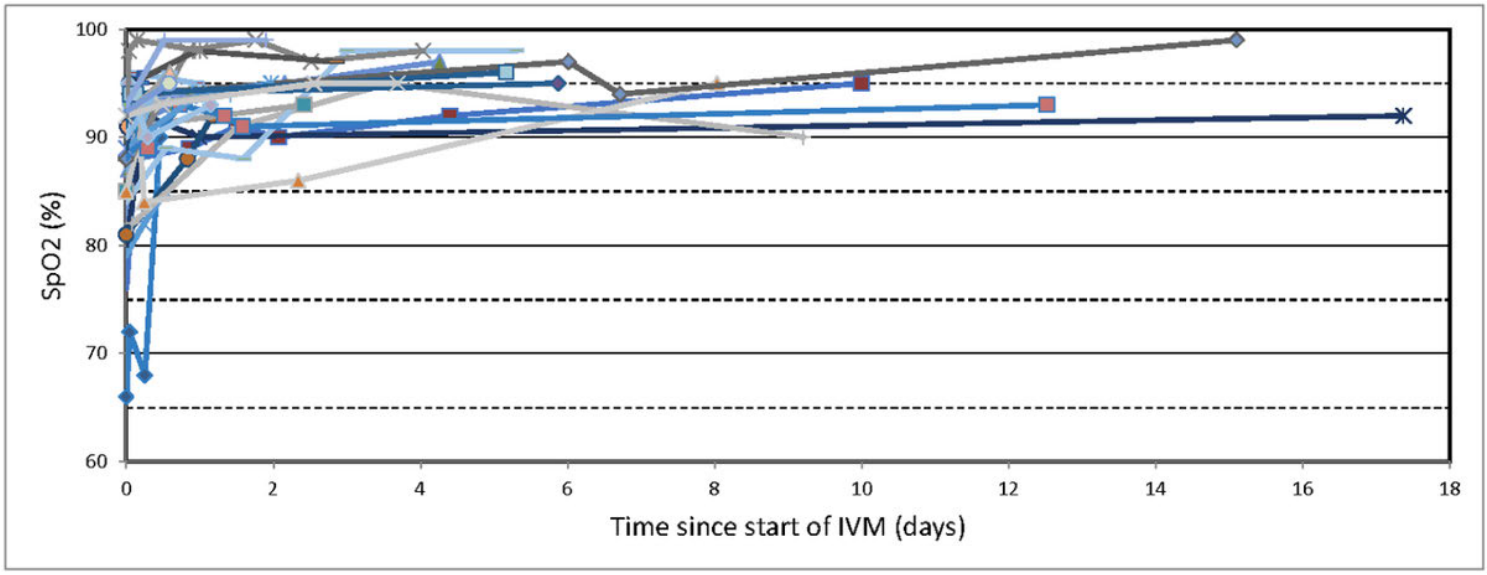


Figure 3

SpO2 values, all on room air, tracked for 34 severe COVID-19 patients treated with IVM, doxycycline and zinc.



Figure 4

Changes in SpO2 over 3 hours for a COVID-19 patient after administration of IVM. SpO2 values are shown (all on room air), with pulse rates (bpm) below those, at pre-treatment (A) and for 45 minutes (B), 90 minutes (C), and 3 hours (D) after first dose of IVM. This patient was diagnosed with COVID-19 by a positive result from a rapid antigen test and treated with combination therapy including IVM, doxycycline and zinc plus other oral and nebulized adjunct agents from among those itemized above.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- JCStoneetal.2021.RapidincreaseofSpO2onroomairfor34severeCOVID19patients.Supplementarytables.pdf

FOI 26-3132 - Document 5

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Changes in SpO2 on Room Air for 34 Severe COVID-19 Patients after Ivermectin-Based Combination Treatment: 62% Normalization within 24 Hours

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Abstract: The emergence of COVID-19 in March 2020 challenged Zimbabwe to respond with limited medical facilities and therapeutic options. Based on early clinical indications of efficacy for the macrocyclic lactone, Ivermectin (IVM), against COVID-19, IVM-based combination treatments were deployed to treat it. Oxygen saturation (SpO2) data were retrospectively analyzed for 34 severe, hypoxic COVID-19 patients all on room air (without supplemental oxygen). The patients, median age 56.5, were treated at clinics or at home between August 2020 and May 2021. All but three of these 34 patients had significantly increased SpO2 values within 24 h after the first IVM dose. The mean increase in SpO2 as a percentage of full normalization to SpO2 = 97 was 55.1% at +12 h and 62.3% at +24 h after the first IVM dose (paired *t*-test, $p < 0.0000001$). These results parallel similar sharp, rapid increases in SpO2, all on room air, for 24 mostly severe COVID-19 patients in the USA (California) who were given an IVM-based combination treatment. All patients in both of these critical series recovered. These rapid increases in SpO2 values after IVM treatment stand in sharp contrast to declines in SpO2 and associated pulmonary function through the second week following the onset of moderate or severe COVID-19 symptoms under standard care.

Keywords: COVID-19; SARS-CoV-2; spike protein; ivermectin; doxycycline; SpO2

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Citation: Stone, J.C.; Ndarukwa, P.; Scheim, D.E.; Dancis, B.M.; Dancis, J.; Gill, M.G.; Aldous, C. Changes in SpO2 on Room Air for 34 Severe COVID-19 Patients after Ivermectin-Based Combination Treatment: 62% Normalization within 24 Hours. *Biologics* **2022**, *2*, 196–210. https://doi.org/10.3390/biologics2030015

Academic Editor: Seth H. Pincus

Received: 10 February 2022
Accepted: 19 May 2022
Published: 31 August 2022

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1. Introduction

Evaluating the efficacy of COVID-19 therapeutics is challenging, since most patients typically recover; the worldwide cumulative case fatality rate was 2% as of September 2021 [1]. Thus, randomized clinical trial (RCT) results are important, yet interpretations, for example, of the mixed results from more than 25 RCTs for COVID-19 treatment with the drug Ivermectin (IVM) [2–5], including three recent studies with negative results [6–8], have been controversial. Yet, such conflicting results have emerged even among RCTs having large patient cohorts, for example those for remdesivir, for which opposite conclusions were drawn by the U.S. FDA (positive) [9] and the World Health Organization (negative) [10] as to efficacy against COVID-19.

Against the backdrop of these mixed RCT results for IVM, a quantifiable determination of whether rapid, major improvements in pulmonary function are achieved shortly after IVM administration to COVID-19 patients can provide an additional means to study efficacy. Such a route of investigation offers additional potential insights when conducted in conjunction with important new studies that indicate potential biological mechanisms by

FOI 26-3132 - Document 5

which IVM could rapidly neutralize the morbidity of a SARS-CoV-2 infection [11,12]. The simplest indicator of lung function is blood oxygen saturation level, SpO2, measured with a pulse oximeter. However, the administration of supplemental oxygen to any COVID-19 patient would preclude meaningful pre- and post-treatment comparisons of SpO2 values. For this retrospective study conducted in Zimbabwe, ironically, challenges that constrained treatment capabilities also provided the opportunity to track changes in SpO2 values, all recorded on room air (without supplemental oxygen), for 34 hypoxic, severe COVID-19 patients within 12 to 48 h after beginning IVM-based combination treatment (see Figure 1). Details of the inclusion and exclusion criteria applied that resulted in the selection of these 34 patients are provided in the Materials and Methods Section below. It should be noted that while inferences can be drawn as to these changes in SpO2 values over a 48 h period following administration of ivermectin, as detailed below, other conclusions are limited by a lack of control groups, randomization, or follow-up.

Zimbabwe, a landlocked country in Southern Africa that shares a border with South Africa, had its first reported case of COVID-19 in March 2020 [13]. Eight cases and one death from COVID-19 followed in the same month [14]. COVID-19 wards were created at a general practice clinic by converting two staff rooms into a four-bed ward and a storeroom into a two-bed ward. Available equipment included several oxygen cylinders, an oxygen concentrator, six beds, and three monitors for SpO2 and blood flow parameters. The staff consisted of the lead author, another primary care physician, and either one or two nurses at different times, each on 12 h shifts. During the initial months of the pandemic, in the absence of proven therapies and protocols, the standard of care evolved through early August 2020 to include corticosteroids, anticoagulants, and antibiotics, as used elsewhere in the world during this period.

However, the efficacy of these treatments was found to be limited, and by the end of July 2020, several COVID-19 deaths were recorded in the country. Based on reports of initial success using IVM for COVID-19 treatment from colleagues in Johannesburg, South Africa, the College of Primary Care Physicians of Zimbabwe (CPCPZ) adopted and included IVM in their COVID-19 treatment protocol from 8 August 2020, starting initially with a 10–12 mg stat dose. Treatment of COVID-19 with IVM continued after the first patients showed improved outcomes, with more rapid recoveries achieved at doses higher than the standard of 200 ug/kg, as initially used. In August 2020, after it became apparent that IVM added to standard of care was significantly reducing the death rate, together with the hospital system being overwhelmed, CPCPZ physicians decided to treat COVID-19 patients where an IVM-based protocol could be administered, including at local general practice clinics that had nursing care and oxygen and at some patients' homes with nursing support and oxygen supplementation as available.

IVM for COVID-19 Treatment

The decision to include IVM in COVID-19 treatment protocols in Zimbabwe was made as the pandemic swept through that nation, overwhelming limited clinical care facilities, with no drug developed to treat COVID-19 being generally accepted as effective. A published case–control study of IVM treatment for COVID-19 conducted at four U.S. hospitals [15] that had been initially released in a preprint in June 2020 found a 40% reduction in mortality among 173 patients treated with low-dose IVM vs. 107 case-matched controls (15% vs. 25.2% deaths). Interest in IVM was supported by its Nobel prize-honored pedigree and its extensive use to treat a variety of human diseases in over 3.7 billion doses worldwide since 1987 [2,16,17]. Another favorable characteristic of this drug is its extraordinary record of safety, well tolerated at high doses [18,19], including in studies for COVID-19 treatment [6,20]. It is generally non-toxic even at doses far exceeding the therapeutic range [21,22]. Since August 2020, inpatient and outpatient treatments of COVID-19 with IVM have been applied across 25 countries [2], with more than 20 RCTs conducted for IVM treatment regimens [2–5].

FOI 26-3132 - Document 5

A comprehensive review of clinical studies for IVM treatment of COVID-19 by the Nobel co-laureate for IVM, Dr Satoshi Omura, and colleagues, concluded that IVM yielded major reductions in mortality [2]. As noted above, however, three recent RCTs for IVM treatment of COVID-19 had negative results [6–8]. However, one of these had multiple protocol violations, with 38 doses of IVM mistakenly substituted for placebo and blinding violated with a glucose placebo [6,23], while related concerns were raised for another [7,24]. Two animal studies of IVM treatment at low human-equivalent doses, one for the SARS-CoV-2 virus in golden hamsters [25] and another for a related betacoronavirus (MHV-A59) in mice [26], found statistically significant treatment benefits. An indicated key biological mechanism of IVM, competitive binding with the SARS-CoV-2 spike protein [11,12], is likely non-epitope-specific, possibly yielding full efficacy against emerging viral mutant strains.

The demonstrated safety of IVM at much higher than standard doses [6,18–20] allowed the latitude for dose escalation for IVM treatment of COVID-19 over time, and combination therapy centered around IVM plus doxycycline and zinc became the standard COVID-19 treatment protocol used by the CPCPZ. This application of multiple drugs against COVID-19 was based on Zimbabwe's experience with prior infectious diseases, for which early, aggressive use of multiple drugs has been a core treatment principle. The potential efficacy of these adjuncts was later supported by successful clinical trial results with treatments using IVM in combination with doxycycline [27] or with doxycycline and zinc [28]. This combination therapy for COVID-19 has been researched and advanced by Thomas Borody [5], who, in 1990, published the first clinical trial of using a triple therapy of three inexpensive repurposed drugs for *H. pylori* [29], the underlying bacterial cause of peptic ulcers. This triple therapy of repurposed drugs became the worldwide standard of care for peptic ulcers a decade later, after the patents for the palliative drugs Tagamet and Zantac expired, and the discovery of *H. pylori* as the cause of peptic ulcers was honored with the Nobel Prize for Medicine in 2005 [5].

2. Results

Table 1 shows the age group, sex, and range of SpO2 values prior to treatment for the 34 patients in this study. Figures 1 and 2 and Table S1 show the progression of pre-treatment and post-treatment SpO2 values within 48 h after the start of treatment (including first dose of IVM) for the 34 COVID-19 patients of this study, all of whom were treated with IVM, doxycycline, and zinc. All of these patients recovered. SpO2 values are shown at pre-treatment, all recorded within one hour before the start of treatment (first dose of IVM) and within 12, 24, and 48 h after start of treatment. The SpO2 value shown for a given patient at time x is that for the latest post-treatment time $\leq$ x (thus, for example, for a patient having an SpO2 measurement at Hour 17 as the only one in the first 48 h after initial treatment, which would be used as their +24 and +48 h values).

Table 1. Patient age group, sex, and range of pre-treatment SpO2 values.

Age * Group	Sex	Initial SpO2 (%) 66–84	85–89	90–93	Total
25–49					
	Female	2	2	1	5
	Male	2	2	2	6
	Total	4	4	3	11
50–59					
	Female	2	2	1	5
	Male	2	2	2	6
	Total	4	4	3	11
60–69					
	Female	0	1	1	2
	Male	0	4	2	6
	Total	0	5	3	8
70–79					
	Female	0	1	0	1
	Male	1	0	0	1
	Total	1	1	0	2
80+					
	Female	0	1	0	1
	Male	0	1	0	1
	Total	0	2	0	2
TOTAL		9	16	9	34

* Mean age = 55; median age = 56.5.

FOI 26-3132 - Document 5

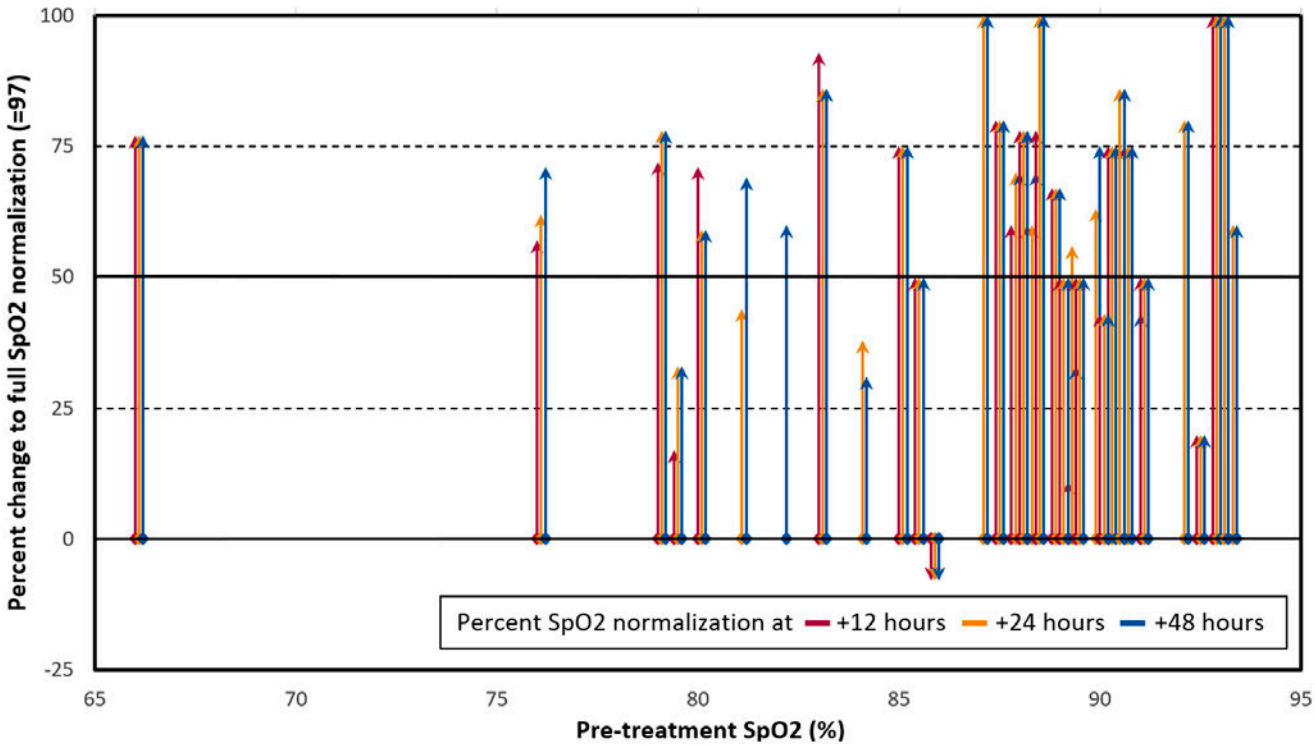


Figure 1. Changes in pre- to post-treatment SpO2 values, all on room air, for 34 severe COVID-19 patients treated with Ivermectin (IVM), doxycycline, and zinc, as percentages of optimal normalization to SpO2 = 97. Red, orange, and blue lines represent, respectively, changes in SpO2 values at +12, +24, and +48 h after the first dose of IVM. Arrows were shifted to the right slightly (<1.5 × axis units), as needed for clarity. For pre- and post-treatment SpO2 values S_0 and S_1, the y axis is the percent of optimal normalization to SpO2 = 97, that is: $100 \times (S_1 - S_0)/(97 - S_0)$, capped at 100%. Mean (±SD) SpO2 changes (y values) were 55.1% ± 28.0% at +12 h, 62.3% ± 26.3% at +24 h, and 64.3% ± 24.5% at +48 h.

Figure 1 shows, for all 34 patients, SpO2 value changes from before IVM administration to post-treatment as percentages of full normalization to an optimal SpO2 value of 97 (95 is considered the minimum normal SpO2 value for a healthy child or adult by the U.S. CDC [30]). Red, orange, and blue lines show, respectively, SpO2 values at +12, +24, and +48 h post-treatment. The mean (±SD) SpO2 changes as this specified percent of optimal normalization were 55.1% ± 28.0% at +12 h, 62.3% ± 26.3% at +24 h, and 64.3% ± 24.5% at +48 h. As shown in Figure 1, the bulk of the patients had 50% or more SpO2 normalization within 48 h, while the sickest 3 (pre-treatment SpO2 ranging from 66 to 78%) improved by over 70%.

Figure 2 shows all pre- and post-treatment SpO2 values grouped into nine graphs (A–I) by the range of pre-treatment SpO2 values and post-treatment times (after first IVM dose) of +12, +24, and +48 h. As shown in Figure 2 and identically in Table 2, those patients with the lowest, mid-range, and highest pre-treatment SpO2 values had mean SpO2 increases at +12 h of 12.8, 5.4, and 2.8, respectively (all SpO2 values in percentage units). Figure 3 shows all pre- and post-treatment SpO2 values throughout the entire observation period for each of the 34 patients. Note that these values were recorded less frequently after a patient's SpO2 value had increased above severe COVID-19 levels (≤93) and general condition had improved.

FOI 26-3132 - Document 5

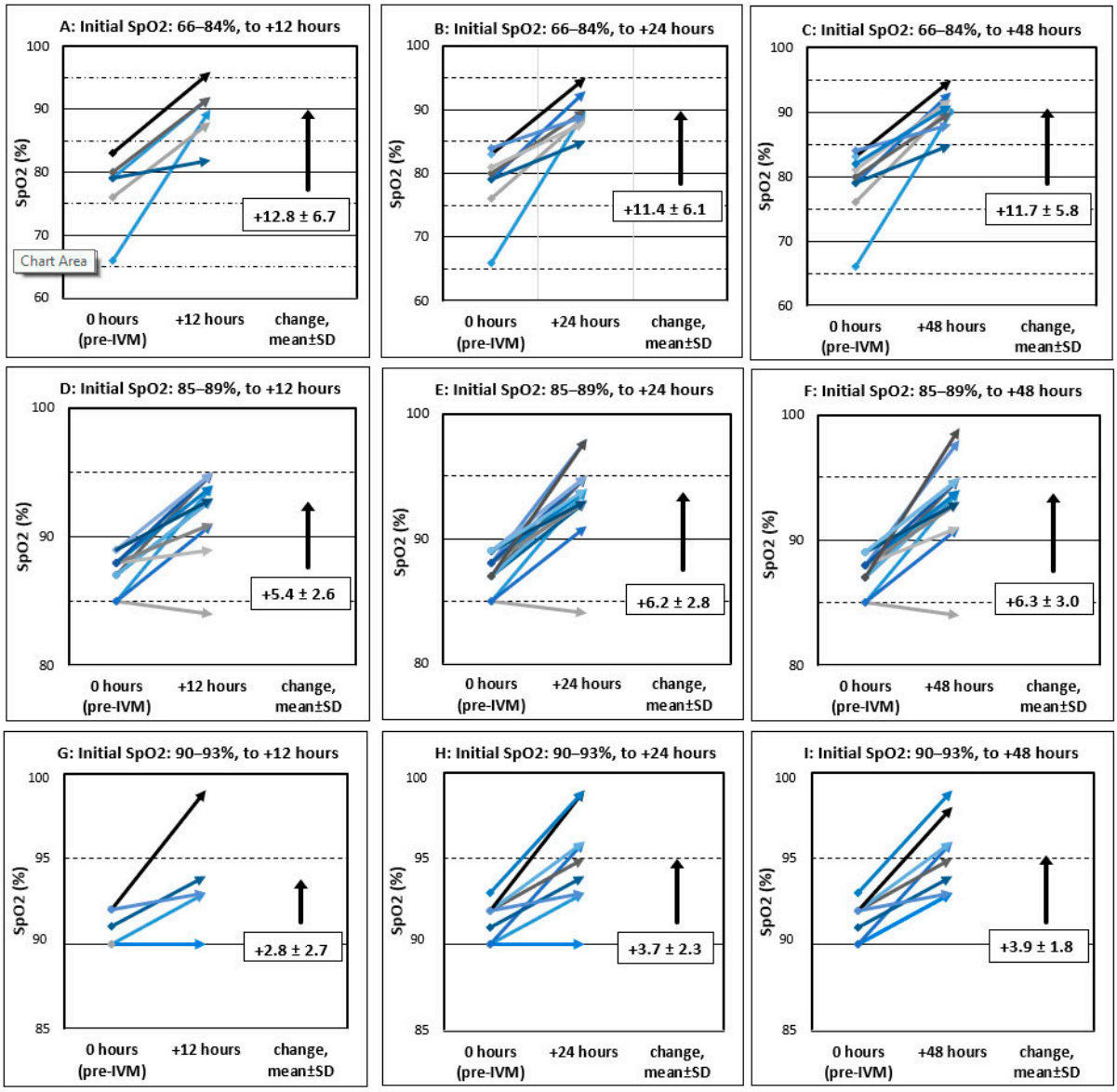


Figure 2. Pre- and post-treatment SpO2 values, all on room air, for 34 severe COVID-19 patients treated with IVM, doxycycline, and zinc. Graphs A–I are in horizontal groupings by the range of pre-treatment SpO2 values and in vertical groupings by elapsed time after first dose of IVM. (The lowest SpO2 value in the stipulated range of 51–93% was 66%.) For these elapsed times of x = 12, 24, and 48 h, the SpO2 value shown for a given patient is at the latest post-treatment time $\leq x$. All pre-treatment SpO2 values are from within one hour before the start of treatment.

Table 2. Mean (± SD) changes in SpO2 values from pre- to post-treatment with IVM-based combination therapy.

	SpO2 (%)		
Pre-Treatment	**at +12 h**	**at +24 h**	**at +48 h**
66–84%	+12.8 ± 6.7	+11.4 ± 6.1	+11.7 ± 5.8
85–89%	+5.4 ± 2.6	+6.2 ± 2.8	+6.3 ± 3.0
90–93%	+2.8 ± 2.7	+3.7 ± 2.3	+3.9 ± 1.8

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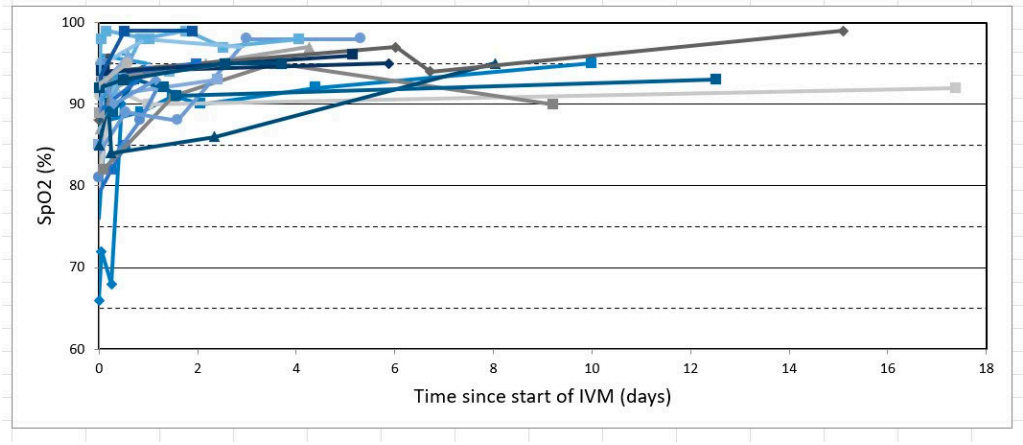


Figure 3. All SpO2 values (on room air) for the 34 severe COVID-19 patients of this study, all treated with IVM-based combination therapy including IVM, doxycycline, and zinc. Each patient is represented by joined line segments of a different color, with markers designating SpO2 values recorded.

Figure 4 shows successive SpO2 values for one patient who had a particularly rapid increase in these values after his first dose of IVM. This patient was a 25-year-old male, treated by a CPCPZ physician at a GP clinic without a supplemental oxygen capability. He received his first 12 mg IVM dose (repeated over the next four days) immediately after entering the clinic with respiratory distress and bilateral pneumonia indicated by stethoscopic examination. His COVID-19 diagnosis was confirmed by a positive result from a rapid antigen test. As shown, his SpO2 values increased from that recorded immediately before treatment (79%) to values at 45 min (87%), 90 min (92%), and 3 h (95%) post-treatment. He was discharged later that same day, and his home SpO2 readings then fluctuated between 92% and 95% over the next three days. By the fourth day after discharge, his SpO2 stabilized at 97%, his pulse dropped to 77 from prior values over 100, and he resumed working from home. Several other patients in this series likewise experienced some degree of tachycardia; however, these were generally manifested prior to the administration of IVM and did not become more pronounced after IVM dosing. While the increases in SpO2 for most of these 34 patients occurred less rapidly, over 48 h, than for this case, as shown in Figures 1–3 and Table S1, several other patients had SpO2 increases of 12% or more within 12 h after the first IVM dose (Table S1): SpO2 76% to 88% for a 38-year-old female; 83% to 96% for a 45-year-old male; 66% to 90% for a 49-year-old female; and 80% to 92% for a 75-year-old male.



Figure 4. Changes in SpO2 over 3 h for a COVID-19 patient after administration of IVM. SpO2 values are shown (all on room air), with pulse rates (bpm) below those, at pre-treatment (**A**) and for 45 min (**B**), 90 min (**C**), and 3 h (**D**) after first dose of IVM. This patient, a 25-year-old male, was diagnosed with COVID-19 by a positive result from a rapid antigen test and treated with combination therapy including IVM, doxycycline, and zinc plus other oral and nebulized adjunct agents from among those itemized above.

FOI 26-3132 - Document 5

As shown in Figure 2, all but two of the 34 patients in this study had increases in SpO2 within the first 48 h after the first dose of IVM. This overall increase in SpO2 continued throughout the entire observation period (Figure 3). Because SpO2 values that are at best stable and typically decreasing for several days after onset of disease symptoms are a well-established norm for moderate and severe COVID-19 patients under standard care, as discussed and shown in Figure 5 and Figure 6 in the Discussion Section below, these sharp, rapid SpO2 increases for all but two of the 34 patients in this study are noteworthy. Furthermore, given this expected change in SpO2 of ≤ 0 within 12, 24, or 48 h after the first IVM dose in the absence of an efficacious intervention against COVID-19, one-tailed paired *t*-test calculations can be performed to assess whether the change in SpO2 values was significantly greater than zero at each time period tracked. Taking into account some missing post-treatment values (see Table S1), there are 25, 33, and 34 pairs of pre- and post-treatment SpO2 values at +12 h, +24, and +48 h, respectively. Applying these paired *t*-test calculations, the SpO2 increases were highly significant for each time period: t = 6.28, $p = 8.5 \times 10^{-7}$ at +12 h; t = 8.42, $p = 6.36 \times 10^{-10}$ at +24 h, and t = 8.81, $p = 3.47 \times 10^{-10}$ at +48 h.

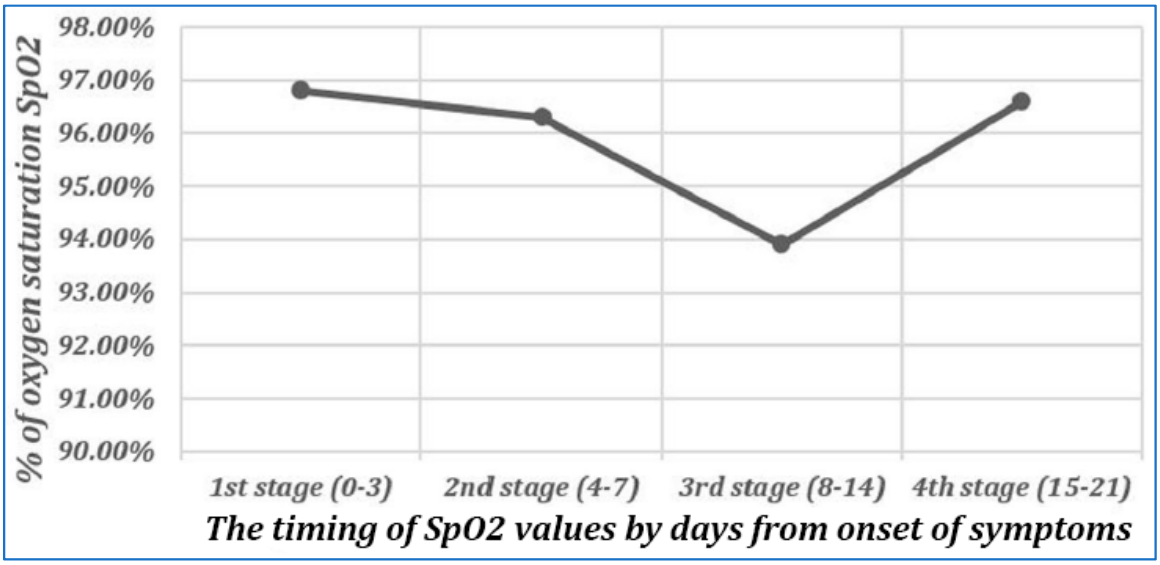


Figure 5. Changes in oxygen saturation during different stages of follow-up of cases with COVID-19 infection. Reproduced from Osman et al., 2020 [31] (CC-BY 4.0). These values were obtained from 125 PCR-confirmed COVID-19 inpatients, March-August 2020, with all values obtained on room air except for 3 patients in Stages 1 and 2, 13 in Stage 3, and 1 in Stage 4. As shown, the mean value of SPO2 decreases from Days 0–3 to Days 8–14; it is inversely correlated with the severity of pulmonary CT abnormalities in these patients throughout the full period of observation.

No serious adverse effects (AEs) from IVM treatment were observed in any patient, although transient AEs such as blurred vision characteristic of higher-dose IVM administration were observed in some patients given doses as high as 100 mg. While comparative results using higher vs. lower doses were not systematically tracked, the practice of increasing IVM doses for patients not initially responding to treatment had generally successful outcomes and became the standard protocol after December 2020.

3. Discussion

This study is a retrospective review of clinical data collected amid the challenges of providing treatment with limited facilities and resources to COVID-19 patients with severe disease. Under such conditions, it was not possible to obtain blood test values for all patients, including values for lymphocyte count, LDH, D-Dimer, and CRP, which were thus not analyzed. On the other hand, the lack of availability of oxygen supplementation for

FOI 26-3132 - Document 5

many of the patients treated in Zimbabwe provided the rare opportunity to track changes in SpO2 values all recorded on room air before and after administration of IVM for this group of 34 severe COVID-19 patients, 74% of them presenting with SpO2 values below 90% and 29% presenting with SpO2 85% and below.

It should be noted that this retrospective review is limited in its conclusions by the lack of control groups, randomization, or follow-up. Although, as noted, all of these 34 severe COVID-19 patients in this series survived, no conclusions as to mortality benefits can be drawn due to the absence of randomized controls. The one clinical parameter for which conclusions can be reasonably drawn is for the change in SpO2 values during the course of a 48 h period following IVM administration, since as detailed below, there is an extensive set of consistent baseline data on changes in SpO2 during the course of a COVID-19 infection under standard care.

The mean ($\pm$SD) SpO2 normalization, as reported above, was 55.1% $\pm$ 28.0% at +12 h, which rose to 62.3% $\pm$ 26.3% at +24 h and then to 64.3% $\pm$ 24.5% at +48 h after the first IVM dose. Paired *t*-test calculations yield $p < 0.0000001$ for each of the SpO2 increases, at +12, +24, or +48 h, having occurred by chance if the IVM-based combination therapy applied had no clinical activity against COVID-19. These highly significant p-values are based on the well-grounded assumption of expected change in SpO2 values of $\leq$0 during this time period. Moderate and severe COVID-19 patients under standard care typically manifest decreasing SpO2 values in tandem with increasing pulmonary CT abnormalities from the day of onset of disease symptoms through the second week following, as established in several studies that tracked SpO2 values, pulmonary abnormalities, or both [31–37]. For example, Figure 5 shows SpO2 values recorded over three weeks for COVID-19 patients under standard care [31], with decreasing SpO2 values through Stage 3, 8–14 days after the onset of symptoms.

The most useful baseline of SpO2 values for comparison would be those on room air for COVID-19 patients having pre-treatment SpO2 values $\leq$ 93% (severe COVID-19), as obtained in this study. Yet such SpO2 data are rarely obtained, since severe COVID-19 patients would typically receive supplemental oxygen. However, results without supplemental oxygen were provided in one study of COVID-19 patients in Nigeria (Thairu et al., 2022), which tracked SpO2 values prior to standard treatment and for ten days following [38] as compared with results of a prior study in which IVM treatment was provided in a similarly scheduled regimen [39]. The patients in the standard treatment series were given varying combinations of lopinavir/ritonavir (Alluvia), remdesivir, azithromycin, and enoxaparin (low-molecular-weight heparin, administered to all patients), along with zinc sulfate and vitamin C. The standard treatment regimen used by Thairu et al., 2022, was aggressive; some studies using just heparin as a mainstay of COVID-19 treatment reported successful results [40–42]. In a molecular modeling study that screened 100 agents [43], heparin was found to have binding affinity to the SARS-CoV-2 spike protein second highest to that of IVM, indicating the potential for heparin, like IVM, to inhibit virally caused morbidities affecting blood oxygenation efficiency [12].

The results for Thairu et al., 2022, its standard treatment group, as shown in Figure 6, are for those 26 COVID-19 patients who had pre-treatment SpO2 values $\leq$ 93% (severe COVID-19), and who were tracked only on room air per limitations on availability of supplemental oxygen [38,44]. Mean SpO2 remained at or below its pre-treatment value through Day 4 and then began to increase at Day 5. Despite the potentially greater than typical improvements in SpO2 for patients under standard care in Thairu et al., 2022, given use of enoxaparin, mean SpO2 did not increase through Day 4, as shown in Figure 6, in contrast to the sharp increases of SpO2 values through Day 2 for our study's 34 patients.

FOI 26-3132 - Document 5

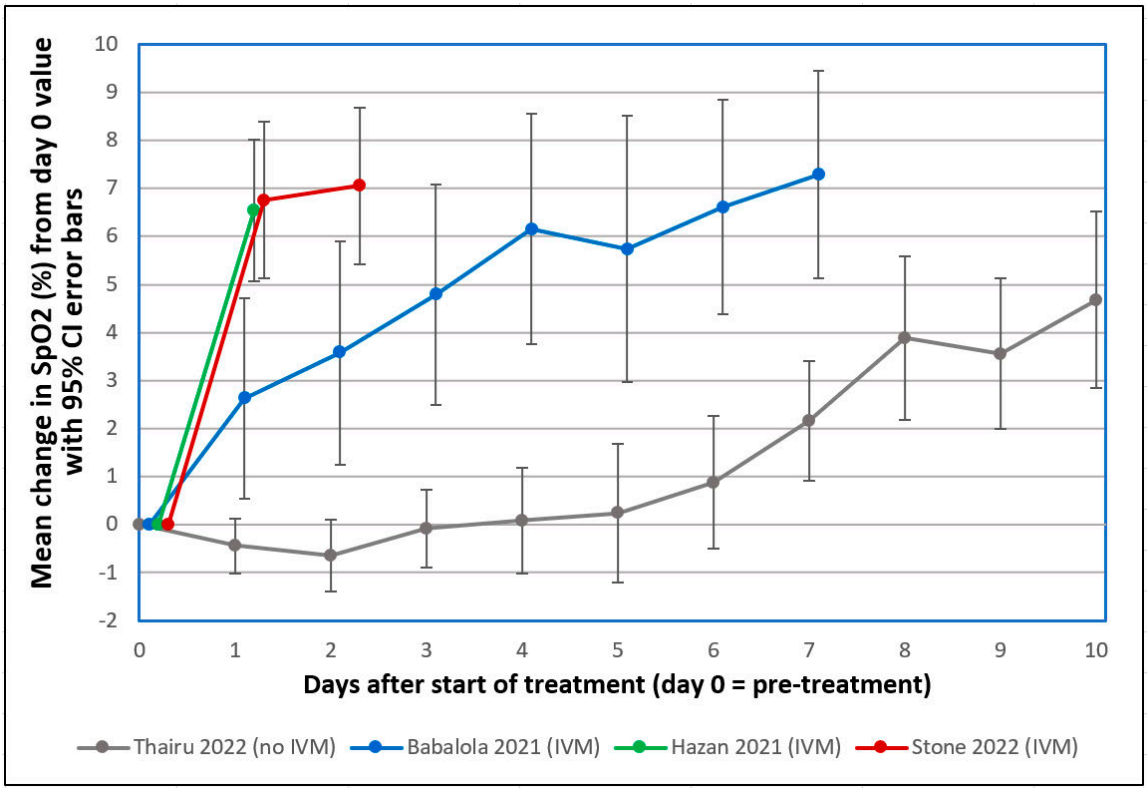


Figure 6. Mean changes in oxygen saturation (SpO2) for severe COVID-19 patients following treatments including or excluding IVM. Patients tracked over various time periods from each regimen were those with SpO2 values all recorded on room air, having pre-treatment (day 0) values $\leq$ 93%. The y axis value at day n is the mean of changes in SpO2 values from day 0 to day n, with error bars designating 95% confidence intervals. • Thairu et al., 2022 [38,44]: 26 patients, median age 45, treated with varying combinations of lopinavir/ritonavir (Alluvia), remdesivir, azithromycin, and enoxaparin plus zinc sulfate and vitamin C. • Babalola et al., 2021 [39,44]: 19 patients, median age 33, treated with IVM, zinc and vitamin C, with some also given azithromycin and hydroxychloroquine. • Hazan et al., 2021 [45]: 19 patients, median age 63, treated with IVM, doxycycline and zinc. • Stone et al., 2022 (this study): 34 patients, median age 56.5, treated with IVM, doxycycline and zinc, as described.

Our study's results, including the recoveries of all 34 patients, parallel those recently reported by Hazan et al., 2022, for which SpO2 values all on room air for 24 RT-qPCR confirmed COVID-19 patients in California (USA) who were tracked before and +24 h after treatment with IVM, doxycycline, and zinc [45]. For the 19 severe COVID-19 patients in that series who had pre-treatment SpO2 values of 90% or below (minimum = 77%) and had post-treatment SpO2 values recorded at +24 h (see Table S2), the mean ($\pm$SD) SpO2 values rose from 86.7% $\pm$ 4.5% pre-treatment to 93.3% $\pm$ 2.6% at +24 h after the first IVM dose. Per our calculation of the percentage of normalization to SpO2 = 97, the mean ($\pm$SD) relative SpO2 increase at 24 h post-treatment for these 19 patients was 65.2% $\pm$ 17.5%, which is close to the mean ($\pm$SD) of 62.3% $\pm$ 26.3% for the 34 patients in our study. The one-tailed paired *t*-test for these increased SpO2 values in these 19 patients yielded t = 9.34, $p < 0.0000001$, which, as for our Zimbabwe study, is highly significant.

In a third study, Babalola et al., 2021, for 19 severe COVID-19 patients in Nigeria, SpO2 values increased significantly one day after the first dose of IVM ($p < 0.008$, one-tailed paired *t*-test) [39,44]. SpO2 values increased even more markedly at two days after the first dose of IVM ($p < 0.0023$, one-tailed paired *t*-test). All of the patients in the Hazan and Babalola studies recovered (38 of these 43 total patients having severe COVID-19 status, as noted), as did all our Zimbabwe patients.

FOI 26-3132 - Document 5

The similar results for the 34 severe COVID-19 patients in our Zimbabwe SpO2 series and the 19 severe COVID-19 patients of Hazan et al., 2022 [45], are both sharper than the significant SpO2 increases obtained by Babalola et al., 2021 [39] and suggest that the triple therapy of IVM, doxycycline, and zinc provides enhanced efficacy with respect to IVM used with other adjuncts. One limitation of our study is that except for nebulized nano-silver, used for all patients at the start of the treatment regimen, other adjuncts were deployed on a case-by-case basis, per the patient's condition. Furthermore, IVM dosages were increased during the study period based on the observations that no serious AEs were observed at higher doses, and higher doses appeared to be more effective for the patients with the most severe symptoms. A follow-up clinical study of IVM-based combination treatment of COVID-19 would benefit from a more structured specification and tracking of dosages and adjuncts used.

The distinct improvements in respiratory function for our study parallel those reported by Hazan et al., 2022, and Babalola et al., 2021, and provide a quantifiable demonstration of rapid clinical improvement in severe COVID-19 patients after IVM treatment. Such rapid improvements had been observed since the first major clinical trial in Florida, USA, of IVM treatment of COVID-19 [15]. The lead investigator of that clinical trial had observed that stabilization and then improvement in breathing function along with blood oxygenation as tracked by SpO2 frequently occurred within 12–48 h after IVM treatment, even for patients who had been deteriorating rapidly and had required supplemental oxygen at up to a 50% mixture [46]. That Florida study also reported a 40% reduction in mortality in the IVM-treated group compared with propensity-matched case controls. Likewise, for a larger series of 92 severe COVID-19 patients treated in Zimbabwe with IVM, doxycycline, and zinc and other agents, the number of case fatalities (two) and deteriorations followed by recoveries (two) were much lower than predicted for a comparable set of patients using a well-regarded risk assessment model [47]. Yet, this risk model assessment is suggestive only, and as stated earlier, no conclusions regarding mortality or deteriorations can be drawn given the lack of controls in this study.

The SpO2 increases within a day after IVM treatment observed in this study and by Hazan et al., 2022, and Babalola et al., 2021, offer a distinct indication that IVM yields rapid, directly observable resolution of pulmonary dysfunction as tracked by SpO2 values for COVID-19 patients. This finding offers clues as to the potential biological mechanism of IVM activity against SARS-CoV-2 since, for example, even an effective freeze on viral replication or rapid repair of damaged pulmonary alveoli would be unlikely to yield such rapid clinical improvements. One indicated biological mechanism of IVM activity, competitive binding with the SARS-CoV-2 spike protein [11,12,48], may act quickly, through a reversal of virally induced blood cell clumping, to increase pulmonary capillary flow and, in turn, account for normalization of blood oxygenation. Another plausible mechanism of IVM activity, based on strong binding affinity of IVM to the alpha 7 cholinergic receptor a7nAChR, as shown in silico [11], is the activation of the cholinergic anti-inflammatory pathway under the control of the vagus nerve [49], which is regulated by acetylcholine and potentiated by the high-affinity binding of IVM (a positive allosteric modulator) to a7nAChR [50] expressed on bronchial, vascular, as well as to cytokine-producing cells (i.e., TNF, IL1- and IL6-secreting macrophages, lymphocytes, and mast cells) [11,51]. Through the stimulation of a related a7nAChR pathway, IVM may also be able to induce vasodilation [11].

It should be noted that similar rapid normalizations of SpO2 deficits were observed in this study and Hazan et al., 2022 [45], which both used IVM, doxycycline, and zinc; and in Rajter et al., 2020, conducted in Florida [15], which used IVM added to the standard of care, compared with controls using the standard of care without IVM. In Babalola et al., 2021 [39], similar rapid increases in SpO2 were observed using IVM, zinc, and vitamin C, as well as hydroxychloroquine and azithromycin for some patients (no significant differences in outcomes were found with or without those two additional agents; the results with and without those two added agents are combined in Figure 6). Although it is reasonable to deduce that since IVM was the common agent in all these studies and the biological

FOI 26-3132 - Document 5

mechanisms noted provide a reasonable explanation for their reported rapid increases in SpO2, without appropriate controls, it is impossible to positively conclude that it was IVM and not various combinations of other adjuncts that caused the SpO2 increases observed.

For our study, rapid increases in SpO2 values after the start of IVM-based combination treatment occurred for all but two of these 34 patients and resulted in recoveries for all of them. As noted above, no serious adverse effects (AEs) from IVM treatment were observed in any patient, and while comparative results using higher vs. lower doses were not systematically tracked, the practice of increasing IVM doses for patients not initially responding to treatment had generally successful outcomes.

4. Materials and Methods

This study is a retrospective review of clinical data collected for 34 severe COVID-19 patients for whom SpO2 values were monitored, all on room air, during the course of their treatment, with therapeutic agents selected by their physicians to offer the greatest chances for clinical benefits and recovery. Clinical data were collected for additional patients in Zimbabwe for whom SpO2 values on room air were not recorded, thus not fitting the inclusion criteria of this analysis, with mortality and deterioration outcomes for that larger patient series having been previously reported [47].

4.1. Outcomes

Outcomes tracked were changes in SpO2 values from within one hour before treatment to 12, 24, and 48 h after start of treatment (first dose of IVM), for 34 COVID-19 patients for whom a pre-treatment SpO2 value and at least one SpO2 value up to +48 h after start of treatment were available, all obtained on room air.

4.2. Participants

The 34 COVID-19 patients of this study were from Harare, treated by CPCPZ physicians either at local clinics or at patients' homes.

4.3. Inclusion and Exclusion Criteria

Patients selected for analysis were of age 18 or older and had treatment start dates between 8 August 2020 and 31 May 2021. Patients selected had an SpO2 value on intake of 51–93%, with all SpO2 values recorded and documented to have been obtained on room air (in almost every case because oxygen was not available), and at least one SpO2 value obtained within 48 h after IVM administration. These patients thus fit the U.S. National Institutes of Health's definition of severe COVID-19, a sufficient condition of which is an SpO2 value of 93% or below [52]. Patients were required to have been found COVID-19 positive either by a positive PCR test (real-time PCR, nasal-pharyngeal swabs) or a clinical diagnosis made by criteria including exposure to a COVID-19 patient, hypoxia, lymphopenia, monocytosis, elevated LDH, elevated dimer, and/or radiology consistent with pulmonary abnormalities caused by the virus. PCR testing was not performed for all patients because, in addition to having significantly depressed SpO2 values $\leq$ 93%, several patients had other symptoms of serious concern and required immediate treatment before the results of a PCR test could be obtained.

4.4. Treatment

All patients were administered an IVM-based combination therapy also including doxycycline and zinc plus selected other agents from the standard of care used prior to August 2020. These agents used in combination with IVM included corticosteroids, clopidogrel, aspirin, enoxaparin, a nebulized nano-silver preparation (applied for all patients), rivaroxaban, zinc sulfate, azithromycin, doxycycline, and, in some cases, an IV antibiotic. Patients treated in a clinic were assessed by a nurse upon admittance, with blood drawn and PCR tests conducted as feasible given the patient's condition and with severe symptoms necessitating immediate treatment. For those patients who contacted a CPCPZ physician

FOI 26-3132 - Document 5

from home requesting treatment, an online questionnaire was first completed by the patient, after which, if COVID-19 was still a suspected diagnosis, a nurse visit to the home was conducted, with associated follow-up per the procedures described for in-clinic patients.

As evidence of IVM safety and tolerability accrued following its use beginning in August 2020, its stat dose of 10 mg as used for the earliest patients was increased on 11 September 2020 to 10–12 mg every four days for three doses. Subsequently, the dosage was further increased to 12 mg IVM on the day of admission and then on Days 4 and 8 plus doxycycline (100 mg b.i.d.) and zinc sulfate (60 mg/day). The latter regimen was used up through December 2020, when the second pandemic wave emerged in Zimbabwe. At that time, additional evidence of the safety and tolerability of this regimen supported further dose escalation to a standard IVM dose regimen of 12 mg daily for five consecutive days, with adjunct use of doxycycline and zinc sulfate continued at the doses noted. In some cases, for which this standard treatment regimen did not yield significant clinical gains within a few days, even higher doses of IVM were used, in some cases as high as 100 mg for a single dose. Transient adverse effects (AEs) such as blurred vision characteristic of high-dose IVM often occurred at those dose levels, but no serious AEs associated with IVM were manifested in any patient.

4.5. Data Collection

SpO2 values were tracked using monitors that continually displayed readings for pulse rate and blood pressure and waveform images of blood pulses. For those patients treated at home, the intake nurse provided a pulse oximeter to the patient, unless the patient had one that the nurse deemed of reliable quality. The patient or a family member took SpO2 readings regularly, using the same oximeter as used for the pre-treatment reading. In most cases, these readings were taken daily, with much lower frequency after SpO2 values had risen significantly and the patient's clinical condition had correspondingly improved. Patients were instructed to message the nurse immediately if SpO2 ever decreased from a higher value to 93% or below or if any new clinical symptoms of concern developed.

4.6. Analytical Methods

For the 34-patient severe COVID-19 patients of this study, a measure of percent normalization toward a fully optimal SpO2 value of 97 was applied, which for pre- and post-treatment SpO2 values S_0 and S_1, was: $100 \times (S_1 - S_0)/(97 - S_0)$, capped at 100%. Pre- and post-treatment SpO2 values are plotted for all 34 patients using this percent normalization measure in Figure 1 and are also presented directly in Figures 2 and 3 and Table S1.

5. Conclusions

Marked, rapid normalizations of blood oxygenation, $p < 0.0000001$ (paired *t*-test), were obtained in each time period analyzed for the 34 severe COVID-19 patients in this study, all treated with IVM-based combination therapy. These results parallel similar rapid SpO2 increases reported by Hazan et al., 2022, for 19 severe COVID-19 patients having pre-treatment SpO2 ≤ 90, also at $p < 0.0000001$, with all SpO2 values likewise on room air. Similar results for IVM treatment were also obtained by Babalola et al., 2021, for 19 severe COVID-19 patients, with paired *t*-test values of $p < 0.008$ at Day 1 and $p < 0.0023$ at Day 2. These sharp, rapid increases in SpO2, accompanied by the survival of all patients in this Zimbabwe study and the California (Hazan) and Nigeria (Babalola) clinical series stand in sharp contrast to the, at best, stable and typically decreasing SpO2 values for several days after the onset of moderate or severe COVID-19 symptoms, even under aggressive non-IVM-based treatment regimens. These SpO2 increases rapidly following the first IVM dose establish a cause-and-effect clinical benefit for IVM-based combination treatment of this disease.

For the 34 patients of this study, treatment at home or in clinics with basic facilities freed up hospital resources for other patients, and the treatment approach modeled in these

FOI 26-3132 - Document 5

studies could significantly relieve the pressure on overwhelmed health facilities. IVM is widely available worldwide, inexpensive, and one of the safest drugs in modern medicine, with its safety in "improving the health and wellbeing of millions" noted explicitly by the Nobel Committee in awarding its 2015 prize for the discovery of IVM [53]. These study results, therefore, support the extended deployment of IVM-based combination treatment for COVID-19, complementary to immunizations for prevention.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biologics2030015/s1, Table S1: Changes in SpO2 for 34 COVID-19 patients treated with IVM, doxycycline, and zinc.; Table S2: Changes in SpO2 for 19 COVID-19 patients treated with IVM, doxycycline, and zinc as reported by Hazan et al., 2022.

Author Contributions: Conceptualization, J.C.S., P.N. and M.G.G.; methodology, J.C.S., M.G.G. and C.A.; investigation: J.C.S. and P.N.; software, B.M.D. and D.E.S.; validation, B.M.D., J.D. and D.E.S.; formal analysis, B.M.D., J.D. and D.E.S.; writing—original draft preparation, J.C.S., D.E.S. and C.A.; writing—review and editing, B.M.D., J.D., P.N. and D.E.S.; project administration, J.C.S. and P.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of The Medical Research Council of Zimbabwe (Protocol Code E293, 23 June 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data underlying the study cannot be made publicly available on an unrestricted basis due to ethical concerns about patient confidentiality. Data will be made available to qualified researchers upon request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AE	Adverse effect
CPCPZ	Primary Care Physicians of Zimbabwe
COVID-19	coronavirus disease 2019
IVM	ivermectin
RCT	randomized clinical trial

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FOI 26-3132 - Document 5

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Received: 5 June 2020 | Accepted: 9 June 2020

DOI: 10.1111/dth.13810

FOI 26-3132 - Document 5

DERMATOLOGIC THERAPY WILEY

LETTER

Further aspects of doxycycline therapy in COVID-19

Dear Editor,

In their work, Conforti et al urge experts to broadly investigate the combination of doxycycline and hydroxychloroquine in treating coronavirus disease (COVID-19).[1] The tetracycline antibiotic doxycycline is a nontoxic inhibitor of mitochondrial biogenesis and cellular respiration[2] besides other known pleiotropic properties.[1] Now that the World Health Organization has just halted hydroxychloroquine trials for COVID-19 because of safety reasons, the time has come to find auxiliary compounds so as to give additional benefit to doxycycline.

We also need to take into account that quite a lot of similarities in metabolic pathways of virally infected and cancer cells have been observed.[3] Viruses usually target mitochondria as cellular power houses and various interplays have been detected between viruses and mitochondrial dynamics.[4] Most viruses require aerobic glycolysis as the energy source for replication and its inhibition could attenuate this process.[4]

Vitamin C is a broad-spectrum antiviral agent[5] and an inhibitor of aerobic glycolysis.[2] The combined administration of doxycycline with vitamin C resulted in a robust eradication of cancer stem cells (CSCs) in in vitro experiments by blocking mitochondrial protein translation and ATP production from glycolysis.[2] The addition of azithromycin further boosted CSC clearance.[2]

Sargiacomo et al also proposed the use of doxycycline or azithromycin in COVID-19.[6] Their concept assumes that SARS-CoV-2 prefers chronologically aged, senescent lung cells for binding and replication causing stormy inflammation and subsequent fibrosis, suggesting the application of senolytic drugs such as doxycycline to prevent fibrotic transformation.[6]

I believe that a combined approach regarding CSC elimination[2] may be transmissible into antiviral therapy. Supplementation of doxycycline with the amplifier vitamin C may result in mitochondrial damage of virally compromised cells, the attenuation of immune response by the inhibition of glycolysis in pro-inflammatory immune cells,[7] and a stronger suppression on postinflammatory fibrosis[8] than with doxycycline alone.

In vitro very low concentration of doxycycline is able to block mitochondria,[2] and this antiinflammatory dose is approved up to a 12-month use in rosacea[9] so a prolonged course together with vitamin C may also be an inexpensive, safe, and promising approach in antiviral prophylaxis and treatment.

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FOI 26-3132 - Document 5

Am. J. Trop. Med. Hyg., 84(4), 2011, pp. 517–531
doi:10.4269/ajtmh.2011.10-0285
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Meeting Report

Doxycycline for Malaria Chemoprophylaxis and Treatment: Report from the CDC Expert Meeting on Malaria Chemoprophylaxis

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Abstract. Doxycycline, a synthetically derived tetracycline, is a partially efficacious causal prophylactic (liver stage of *Plasmodium*) drug and a slow acting blood schizontocidal agent highly effective for the prevention of malaria. When used in conjunction with a fast acting schizontocidal agent, it is also highly effective for malaria treatment. Doxycycline is especially useful as a prophylaxis in areas with chloroquine and multidrug-resistant *Plasmodium falciparum* malaria. Although not recommended for pregnant women and children < 8 years of age, severe adverse events are rarely reported for doxycycline. This report examines the evidence behind current recommendations for the use of doxycycline for malaria and summarizes the available literature on its safety and tolerability.

SUMMARY

Indications. The only U.S. Food and Drug Administration (FDA)-approved indication for the use of doxycycline for malaria is for prophylaxis of *Plasmodium falciparum* in short-term travelers (< 4 months) to areas with chloroquine and/or pyrimethamine-sulfadoxine-resistant strains. Although not FDA approved, doxycycline is recommended for prophylaxis of other species of malaria[1] and may be used long term. When used in conjunction with other medications, doxycycline can be used for the treatment of malaria in non-pregnant adults and children ≥ 8 years of age: with quinine sulfate for uncomplicated, chloroquine-resistant *P. falciparum*; with both quinine sulfate and primaquine for uncomplicated chloroquine-resistant *Plasmodium vivax*; and with parenteral quinidine for severe malaria.[2]

Dosing. Available in multiple dosage forms and strengths, doxycycline tablets or capsules of 100 mg are most often used for malaria prophylaxis or treatment. Tablets or capsules should be swallowed with an adequate amount of fluid and should be taken with food. Intravenous (IV) formulation is available for patients unable to take oral medications for treatment. For IV, avoid rapid administration.

Adults. Prophylaxis: 100 mg base once daily starting 1–2 days before travel, then daily during travel, and daily for 4 weeks after leaving the malarious area.

Treatment: 100 mg twice a day for 7 days; must be used in conjunction with a fast acting schizontocide. Primaquine is also required if treating *P. vivax* or *Plasmodium ovale*.

Children. For children ≥ 8 years of age.

Prophylaxis: 2.2 mg/kg (up to adult dose of 100 mg) daily starting 1–2 days before travel, daily during travel, and daily for 4 weeks after leaving the malarious area.

Treatment: 2.2 mg/kg (up to adult dose of 100 mg) twice a day for 7 days; must be used in conjunction with a fast acting schizontocide. Primaquine is also required if treating *P. vivax* or *P. ovale*.

Efficacy. Prophylaxis: Protective efficacy of doxycycline has been shown in three randomized placebo-controlled trials to be between 92% and 96% for *P. falciparum* and 98% for primary *P. vivax* infection.

Treatment: When used in combination with a fast acting schizontocide, treatment efficacy of doxycycline has been shown to be 96–100% in three open-label trials.

Pharmacokinetics. Limited data suggest that milk and other dairy products may limit the bioavailability of doxycycline. It is recommended to separate doxycycline and ingestion of dairy products by 2–3 hours.

Adverse drug reactions. Most common mild/moderate adverse drug reactions (ADRs): nausea, vomiting, abdominal pain, photosensitivity, and vaginitis. Women prone to candidal vaginitis should consider carrying a self-treatment course of an anti-fungal. Severe ADRs are uncommon and include esophagitis and esophageal ulcerations. Doxycycline monohydrate has been observed to have less gastrointestinal side effects than doxycycline hyclate.

Contraindications. Doxycycline is contraindicated in those who have known hypersensitivity to tetracyclines.

Drug interactions. Decreased absorption of doxycycline can occur if taken concurrently with medications with divalent or trivalent cations such as antacids, laxatives, and oral iron preparations. Although the current FDA label states "concurrent use of tetracycline may render oral contraceptives less effective," recent studies have failed to show a significant association between oral contraceptive failure and tetracyclines. Tetracyclines may potentiate the effect of oral anticoagulants, therefore patients on oral anticoagulants should have prothrombin times monitored closely and dose adjusted as needed. Concurrent use of tetracyclines and methoxyflurane anesthesia is not recommended. Barbiturates, carbamazepine, and phenytoin decrease the half-life of doxycycline.

Warnings. Pregnancy category D: Do not use in pregnancy except for the treatment of life-threatening multidrug-resistant *P. falciparum* when no other treatment options are available. Not recommended for children < 8 years of age.

Use during breastfeeding. Excreted in low concentrations in breast milk; noted to be compatible with breastfeeding by the American Academy of Pediatrics.

INTRODUCTION

Doxycycline is a broad-spectrum bacteriostatic agent synthetically derived from naturally occurring tetracyclines produced

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FOI 26-3132 - Document 5

by *Streptomyces* sp. bacteria.[3] Doxycycline was invented and clinically developed in the early 1960s by Pfizer Inc., New York, NY, and marketed under the brand name Vibramycin. Vibramycin received FDA approval in 1967, becoming Pfizer's first once-a-day broad-spectrum antibiotic. The additional indication for malaria prophylaxis was obtained in 1994 with a supplemental New Drug Application. Tetracyclines were first reported efficacious against drug-resistant malaria in the 1970s.[4,5] Early studies examined the effectiveness of tetracycline, minocycline, and doxycycline as antimalarials.[4–8] The evidence for minocycline beyond these early studies is limited, and the four times per day dosing of tetracycline may make adherence difficult. Of the tetracyclines, doxycycline has the most evidence for its effectiveness as an antimalarial.

RECOMMENDED USES AND DOSING OF DOXYCYCLINE

Doxycycline can be used by travelers to all malaria-endemic areas for malaria prophylaxis. When used in conjunction with other medications, doxycycline can also be used to treat malaria.

Prophylaxis. Doxycycline can be used for the prevention of malaria in travelers to malaria-endemic areas and is a good option for areas with chloroquine or multidrug-resistant *P. falciparum*. For prophylaxis, doxycycline is taken once daily beginning 1–2 days before travel, while in malarious areas, and for 4 weeks after leaving. Daily dosage for children ≥ 8 years of age is 2.2 mg/kg (not to exceed the adult dose of 100 mg) and for adults 100 mg.

Treatment. Doxycycline should always be used in conjunction with another drug for treatment of malaria. Quinine with doxycycline can be used for uncomplicated malaria caused by chloroquine-resistant *P. falciparum* or *P. vivax*. When used for severe malaria doxycycline should be used with intravenous quinidine. The treatment dose of doxcycyline in adults is 100 mg twice a day and for children 2.2 mg/kg twice a day up to a maximum of 100 mg twice daily. For those unable to tolerate oral medications, doxycycline is available in a parenteral form and can be made in concentrations between 0.1 and 10 mg/mL. If pain occurs while receiving doxycycline intravenously, it is recommended to use more dilute concentrations (no lower than 0.1 mg/mL) infused at rates no higher than 1 mg/min[9]; switch to an oral formulation once the patient is able to tolerate medications by mouth. If treating *P. vivax* or *P. ovale*, in addition to the aforementioned drugs, primaquine must also be given to prevent relapses (see Pharmacodynamics section).

EFFICACY AND EFFECTIVENESS

The emergence of chloroquine-resistant *P. falciparum* in the 1960s spurred efforts to examine alternative medications such as tetracyclines with antimalarial activity. Early studies examined the treatment and prophylactic efficacy of tetracyclines such as doxycycline, tetracycline, and minocycline against chloroquine-resistant *P. falciparum*. Since these earlier studies, the preponderance of literature on efficacy of tetracyclines as antimalarials has focused on doxycycline.

Overall, studies have showed that doxycycline is a slow-acting blood schizontocidal agent, had partial causal effects and no sporontocidal or gametocytocidal effects, and is effective in the treatment and prevention of malaria (prophylaxis Table 1).[10–18] Doxycycline 100 mg daily has been shown to be highly effective as a blood schizonticidal agent, meaning that it kills the asexual, erythrocytic stages of the malaria parasite.[10,12–21] Three randomized, double-blinded, placebo-controlled studies showed that in semi-immune patients, 100 mg of doxycycline daily had a protective efficacy of 84% (95% confidence interval [CI] = 79.9–97.5%), 92.6% (95% CI = 79.9–97.5%), and 96% (95% CI = 85.4–99.6%) for *P. falciparum* infections.[10,11,18] One of these studies found that doxycycline had a 98% (95% CI = 88.0–99.9%) protective efficacy against primary *P. vivax* infection,[18] but did not examine protective efficacy against relapses. Another randomized, double-blinded, placebo-controlled study showed a 99% (95% CI = 94–100%) protective efficacy of doxycycline 100 mg daily for *P. falciparum* in 67 non-immune Indonesian soldiers.[13] However, in two of these studies, participants were not followed up after leaving the malaria-endemic areas, and it was unclear if prophylactic medications were continued after leaving. One open-label trial followed non-immune patients for 3 months after returning from a malaria-endemic country. Patients had received 100 mg of daily doxycycline while abroad. Of these 171 patients, only 5 (0.6%) developed *P. falciparum* malaria during the follow-up period. Of these 5, one developed malaria while on prophylaxis and 4 developed symptoms after discontinuation of prophylaxis during the 3 months of observation upon returning to a malaria-free country.[12]

Other studies have also shown doxycycline to have inadequate causal activity, or activity against the liver stage of the malaria parasite, which has implications for doxycycline's post-exposure course.[5,22–24] One randomized, double-blinded, placebo-controlled trial examined the efficacy of doxycycline prophylaxis for *P. falciparum* malaria when taken by individuals exposed to mosquitoes infected with *P. falciparum*. Doxycycline was taken daily 3 days before exposure and for 6 days post exposure. Four of 12 persons taking that preventive regimen developed falciparum malaria 15–24 days after completing medications; the protective efficacy was 67% (95% CI = 35–90%).[23] Another study found that of 60 soldiers who received 100 mg of doxycycline a day before deployment, 6 weeks during their deployment, and for 3 days after their return, none had falciparum malaria, whereas two developed vivax malaria.[16] These studies suggest that doxycycline has some tissue schizontocidal activity for *P. falciparum*, but is insufficient for causal prophylaxis. Doxycycline would therefore have to be continued for a sufficient amount of time post exposure for successful suppression of parasites that emerge from the liver. Studies have observed that[5,22–24] malaria developed within 3–4 weeks post exposure. It is therefore recommended that doxycycline prophylaxis be continued for 4 weeks post exposure.

Doxycycline's limited activity in the liver stages of *Plasmodium* also means that it does not kill *P. vivax* hypnozoites. Although very effective for preventing primary *P. vivax* infections, doxycycline is not effective at preventing relapses.

The dose of doxycycline required to achieve successful malaria prophylaxis was also examined. The efficacy of half-dose doxycycline (50 mg/day, $N = 67$) was compared with full-dose doxycycline (100 mg/day, $N = 77$). One open-label, randomized, controlled trial found that full-dose doxycycline was significantly more effective in prevention of *P. vivax,*

FOI 26-3132 - Document 5

TABLE 1
Efficacy of doxycycline for prophylaxis*

Country (population)	Study type	Sample size on doxycycline	Duration of prophylaxis	Efficacy (95% CI, if reported)
Kenya (semi-immune)[10]	Randomized, double-blind, comparators = azithromycin daily ($N = 59$), azithromycin weekly ($N = 58$), and placebo	55	10 weeks	Doxycycline: 92.6% (79.9–97.5%) Azithromycin daily: 82.7% (68.5–91.1%) Azithromycin weekly: 64.2% (47.1–77.1%) azithromycin weekly
Kenya (semi-immune, children aged 9–14)[11]	Randomized, double-blind, comparators = primaquine daily ($N = 32$), proguanil daily plus chloroquine weekly ($N = 37$), vitamin daily plus weekly mefloquine ($N = 30$), vitamin alone ($N = 34$)	32	11 weeks	Doxycycline: 84% (66–92%) Primaquine: 85% (68–93%) Mefloquine: 77% (55–88%) Chloroquine plus proguanil: 54% (25–72%)
Gabon and Central African Republic (non-immune)[12]	Randomized, open-label, comparator = CP ($N = 270$)	171	Travel: 4 months	Doxycycline: 97.0% CP: 98.9%
Irian Jaya (non-immune)[13]	Randomized, double-blind, comparator = MQ ($N = 68$) and placebo	67	13 weeks	Doxycycline: 99% (94–100%) overall, 98% (88–100%) for *P. falciparum*, 100% (90–100%) for *P. vivax* MQ: 100% (96–100%) overall, 100% (93–100%) for *P. falciparum*, 100% (91–100%) for *P. vivax*
Thai–Burmese border (semi-immune children aged 10–16 years)[14]	Randomized, open-label, comparators = half-dose doxycycline ($N = 77$), placebo	67	5 months	Full-dose: 5 cases/1070 person-weeks Half-dose: 20 cases/1176 person-weeks
Thai-Burmese border (semi-immune children aged 19015 years)[15]	Randomized, open-label, comparator = CQ ($N = 93$)	95	9 weeks	Doxycycline: 5 cases/597 person-weeks CQ: 31 cases/488 man-weeks
Papua New Guinea (PNG), Malaysia, Thailand (non-immune)[16]	Open-label, comparators = MQ ($N = 40$, PNG), doxycycline + PQ ($N = 69$, PNG), doxycycline + CQ ($N = 125$, Malaysia and Thailand)	60 55	6 weeks 3 weeks	Doxycycline ($N = 60$): 100% for *P. falciparum*, 96.7% for *P. vivax* Doxycycline ($N = 55$): 100% for *P. falciparum*, 76.4% for *P. vivax* MQ: 100% for *P. falciparum*, 90% for *P. vivax* Doxycycline + PQ: 100% for both *P. falciparum* and *P. vivax*
Thai-Kampuchean border (non-immune)[17]	Randomized, open-label, comparators = half-dose doxycycline ($N = 243$) or phyrimethamine + dapsone ($N = 123$), adherence observed but not enforced	243	17 weeks	Doxycycline + CQ: 81% (Adherence 73% for both doxycycline groups)
Irian Jaya, Indonesia (non-immune)[18]	Randomized, double-blind, placebo-controlled, comparators: azithromycin ($N = 148$), placebo	75	20 weeks	Doxycycline: 97.1% (91.2–99.4%) overall, 96.3% (85.4–99.6%) *P. falciparum*, 98.0% (88.0–99.9%) *P. vivax* Azithromycin: 84.7% (75.5–90.7%) overall, 71.6% (50.3–83.8%) *P. falciparum*, 98.9% (93.1–99.9%) *P. vivax*

* CI = confidence interval; CP = chloroquine-proguanil; MQ = mefloquine; PQ = primaquine; CQ = chloroquine.

however the study was not powered to detect a difference between the two doses of doxycycline in preventing *P. falciparum* infections.[14] Another study examined the effectiveness of full-dose and half-dose doxycycline under operational conditions; adherence to medications was not enforced. Although adherence to full versus half-dose doxycycline was not examined, the study found that on average 73% of the doxycycline regimen was taken and that half-dose (33% infected) was significantly less effective than full-dose doxycycline (19% infected) at preventing both *P. falciparum* and primary *P. vivax* infection.[17]

Treatment (Table 2).[5,26–29] Doxycycline has been shown to be a slower acting blood schizonticide during the course of an acute *Plasmodium* infection relative to other antimalarials.[30] However, doxycycline achieves good treatment efficacy when given with rapidly acting shizontocidal drugs such as quinine or quinidine. Studies have reported parasite clearance times (*P. falciparum*) between 3.8 and 6.0 days and fever clearance times between 3.6 and 4.0 days in patients treated with only tetracycline or doxycycline.[4,7,25,26] These clearance times are longer than those observed in patients given quinine alone with parasite and fever clearance times of 2.6 days and 1.9–2.6 days, respectively.[31,32] In terms of treatment efficacy, in patients given doxycycline or tetracycline alone for *P. falciparum*, cure rates between 75% and 100% have been reported.[5,7] Therefore, the slowness with which tetracyclines act make them of limited usefulness as a monotherapy in *P. falciparum* infections.[5,7] The usefulness of tetracycline when given with a faster acting schizontocidal drug, quinine, was studied when decreased effectiveness of quinine was observed in Southeast Asia.[7,32,33] Two open-label trials, one non-randomized and the other randomized, gave subjects quinine (640 mg three times daily for 3 days, and 600 mg four times daily for 7 days, respectively) and tetracycline (250 mg four times daily for 10 days) and found treatment efficacies between 96% and 98% with average parasite clearance times between 3 and

FOI 26-3132 - Document 5

TABLE 2
Efficacy of doxycycline with or without quinine for treatment of *P. falciparum* malaria

Country (population)	Study type	Sample size/doxycycline dose	Duration of treatment	Cure rate (95% CI)
United States (mixture of immune and non-immune)[5]	Challenge, open-label	*P. vivax:* 1/100 mg twice a day 1/100 mg twice a day *P. falciparum:* 4/100 mg twice a day 9/100 mg twice a day	4 days 5 days 7 days	0 0 0 100%
West Malaysia (varied, children aged 2 months–8 years)[27]	Challenge, open-label	9/4 mg/kg daily 26/4 mg/kg daily	4 days 7 days	44.4% 84.6%
Irian Jaya, Indonesia (non-immune)[26]	Randomized, open-label, comparator = CQ (N = 30) and doxycycline + CQ (N = 39)	20/100 mg twice a day	7 days	*P. falciparum*: 64.7% (42.0–87.4%) *P. vivax*: 33.3% (6.6–59.5%)
Brazil (semi-immune)[28]	Randomized, open-label, comparator = AL (N = 28)	31/100 mg twice a day + 500 mg quinine every 8 hours	5 days doxycycline 3 days quinine	100%
Pakistan (unknown immune status)[29]	Challenge, open-label	100/100 mg twice a day + 10mg/kg quinine every 8 hours	7 days doxycycline 3 days	100%

3.6 days and a fever clearance time of 2.6 days.[7,34] Furthermore, one study observed that tetracycline contributed to the maintenance of plasma quinine levels above the minimum inhibitory concentration during the treatment period.[35] It was then extrapolated from these studies that quinine plus doxycycline would also be an effective malaria treatment regimen. More recently, an open-label trial showed 100% cure of all subjects given quinine (10 mg/kg three times a day for 3 to 7 days) and doxycycline (100 mg twice a day for 7 days) for treatment of uncomplicated *P. falciparum* malaria.[29] Therefore, doxycycline should be given with a rapidly acting schizontocide for timely and effective malaria treatment.

PHARMACOKINETICS AND PHARMACODYNAMICS

Pharmacokinetics. An oral dose of 100–200 mg of doxycycline is almost completely absorbed in the duodenum and stomach and is detectable in the blood 15–30 minutes after administration. Following a 200 mg oral dose, peak concentrations of ~2.6 µg/mL are reached at ~2 hours, but this may vary as gastrointestinal (GI) absorption rates can differ among individuals.[3,36,37] Doxycycline, being highly lipophilic, is readily transported across cell membranes, resulting in widespread distribution in body tissues and fluids.[37] Doxycycline binds to serum proteins, localizes in the bone marrow, liver, and spleen, crosses the placenta, and is excreted in breast milk.[3] Tetracyclines also form tetracycline-calcium orthophosphate complexes in sites of calcification such as developing teeth and bone.[3] The bioavailabililty of the doxycycline derivatives, the monohydrate free base and the hydrochloride salt (hyclate), has been shown to be equivalent.[37]

According to the FDA, the absorption of doxycycline "is not markedly influenced by simultaneous ingestion of food and milk," despite the reduced absorption observed with other tetracyclines. However, more recent studies on this subject, while limited, have shown that the absorption of doxycyline is affected to some degree by food and milk, but the clinical consequence of this is not well understood. Food has been shown to decrease serum levels of doxycycline from 4.4 to 4.0 µg/mL, but this has not been shown to be clinically significant.[3,37] Milk decreases the absorption of tetracyclines because of chelation between the calcium in the milk and tetracyclines, however the magnitude of this decrease varies between different tetracycline preparations, and the data for doxycycline is limited.[38,39] One single-dose, cross-over study administered doxycycline with either water or milk to nine volunteers and found that milk decreased the peak plasma drug concentration by 24% and that the area under the plasma concentration-time curve from 0 to 32 hours decreased by 35%.[39] How this decrease in bioavailability translates clinically is not known. Furthermore, doxycycline is a lipophilic drug and it is not known if the fat content of the food or drink affects its absorption. In these studies, the fat content of the food and milk ingested was not discussed. Therefore, food taken with doxycycline to prevent gastrointestinal side effects is not expected to decrease efficacy of the drug; however, there is some limited data that suggests that milk reduces the bioavailability of doxycycline, which may have a negative impact on drug efficacy as in other tetracyclines. Because of absent better data, it would seem prudent to caution individuals to avoid milk and other dairy products when ingesting their daily doxycycline. Separation of the ingestion of doxycycline and ingestion of milk by 2–3 hours (time for absorption and peak concentration) should be satisfactory.

Unlike other tetracyclines, excretion of doxycycline occurs primarily by the gastrointestinal tract and to a much lesser extent by the kidneys.[40–42] Most of a doxycycline dose diffuses from the serosal to the mucosal side of the small bowel. Doxycycline is chelated to an inactive form intraluminally and is excreted.[41,43] A very small portion is also concentrated by the liver in bile for excretion in feces.[41,43] Serum half-life of doxycycline (15–25 hours) is not affected by impaired renal function or hemodialysis, and in patients with renal failure, all excretion of doxycyline occurs by the gastrointestinal route of excretion.[42,43]

There are limited to no data on sex, age, body weight, or race differences in the pharmacokinetics of doxycycline. One study found no differences between males and females (N = 14 males and 14 females).[44]

Pharmacodynamics. Although it is known that doxycyline is a blood schizontocide,[45] the exact mechanism of action is not well defined. In *P. falciparum*, doxycycline has been

FOI 26-3132 - Document 5

observed to block the expression of apicoplast genes, leading to nonfunctional apicoplasts in subsequent progeny, and impeding the development of viable parasites.[46] It is also thought that doxycycline's antimalarial actions may be similar to its bacteriostatic actions of binding to ribosomal subunits and inhibiting protein synthesis, but this has only been observed in supra-pharmacologic doses.[47] Doxycycline has also been observed to have some degree of pre-erythrocytic activity in *P. falciparum*.[16] Of 55 men given a doxycycline prophylaxis regimen that ended 3 days after travel, 13 developed *P. vivax* and none developed *P. falciparum*.[16] Two other studies observed breakthroughs to doxycycline prophylaxis for *P. falciparum* between 2 and 12 weeks after administration of medication and for *P. vivax* between 4 and 9 weeks.[14,15] There are no data on the causal activity of doxycycline for the other species of malaria. On the basis of these studies, the recommended duration of the post-exposure course of doxycycline is 4 weeks. Doxycycline has no effect on the hypnozoites of the *Plasmodium* species.[16,45] Because of this, when treating patients infected with species such as *P. vivax* and *P. ovale* with quinine and doxycycline, a 2-week course of primaquine must be added to kill hypnozoites.[48] There is very limited evidence on doxycycline's effect on gametocytes. Only one study was found that looked at gametocytemia and doxycycline. In this study, *P. vivax* gametocytemia increased after treatment with doxycycline (from 32% pre-treatment to 44% immediately post-treatment) with a median gametocyte clearance time of 62 hours.[49] Although gametocytemia has no clinical implications, malaria in an individual being treated with doxycycline may still be transmitted through mosquitoes.

There is no global consensus on the threshold of susceptibility for use in *in vitro* susceptibility testing for doxycycline. Traditionally, culture-derived or field *P. falciparum* isolates are incubated with varying concentrations of drug and inhibition of replication is measured by several methods to yield a 50% inhibitory concentration (IC50) at 48, 72, or 96 hours. The assay conditions and the drug mechanism of action can markedly affect results.[50] Frequently, the incubation time for these assays is one cycle of parasite replication or 48 hours, but for doxycycline at least 72 hours and preferably 96 hours is needed, depending on the assay method, to obtain accurate results. The increased incubation time is required because doxycyline has been shown to exert its effect during the first 48 hours, but is only detectable in second generation parasites near the 96 hour time point.[46,51,52] However, most studies have been limited to 48-hour assays. One study in French Guiana suggested an IC_{50} cutoff level of 9.6 μM for a 48-hour assay and noted that after both quinine and doxycycline were recommended as first-line malaria treatment in 1995, there was an increase of mean IC_{50s} from 9.6 μM in 1996–1999 to 13.1 μM in 2005.[53] Another study examined *P. falciparum* samples of French patients who traveled to various African countries between 1997 and 2006 using a 42-hour assay and found an overall mean IC_{50} of 9.3 μM with country-specific means ranging between 6.2 and 11.1 μM.[54] The findings of these two studies found many samples with IC_{50s} above the suggested 9.6 μM threshold. However, these findings are not consistent with the relative dearth of reporting of doxycycline failure (when taken appropriately either as prophylaxis or treatment) in the literature. Another study further identified possible molecular markers for decreased *in vitro* susceptibility to doxycycline defined as an IC_{50} of 35 μM.[55]

Resistance of *P. falciparum* to doxycycline is not described but breakthroughs in prophylaxis have been associated with inadequate doses,[14] possibly inadequate serum levels,[11] and poor compliance.[56,57] A clear relationship between the development of clinical symptoms with confirmed *P. falciparum* parasitemia and a serum or plasma level of doxycycline or a predictive IC_{50} in a well-qualified *in vitro* susceptibility assay remains to be determined. More studies are needed to examine emergence of doxycycline resistance and establish a clinically relevant susceptibility threshold.

ADHERENCE

Adherence to daily doxycycline throughout the period of exposure to malaria and for 4 weeks following exposure is vital in achieving full effectiveness of doxycycline as a malaria prophylactic agent. The effectiveness of doxycycline among persons in whom adherence was not enforced was compared with persons in whom compliance was enforced. Effectiveness was only 89% (78–96%) for all types of malaria compared with 99% (94–100%) in soldiers in whom adherence was enforced.[13] Because of doxycycline's short half-life and the mechanism of action (asexual parasite killing at the end of two cycles of erythrocytic replication or 96 hours) relative to that of weekly medications, missing a few doses of doxycycline would have greater consequences than being a few days late for a medication with a long half-life.

Most studies suggest that adherence to daily doxycyline is more challenging than adherence to a weekly medication. One study reported that full adherence to doxycycline daily (81%, $N = 52$) was lower than that for weekly mefloquine (98%, $N = 344$) among United States troops in Somalia; however, it was not discussed whether administration of one or both medications was supervised.[19] Another study in Thai soldiers did not enforce or observe drug administration and reported that adherence (at least 75% of the time) occurred in more than 70% of men given a weekly drug (Maloprim) and in only 50% given doxycycline daily.[17] Survey-based studies of travelers or military personnel have found adherence rates for doxycycline prophylaxis to be between 70% and 84%. A decrease in adherence over time has also been demonstrated. In Australian troops, adherence to daily doxycycline decreased from 60% at 2 months to 44% at 4 months.[21] Only one study of French soldiers in sub-Saharan Africa comparing daily doxycycline to weekly chloroquine-proguanil (CP) found that a decrease in adherence occurred over time in both groups regardless of regimen.[58] Therefore, studies seem to show that short-term adherence to daily prophylaxis is more challenging than for a weekly medication, but long-term adherence decreases for both daily and weekly prophylactic regimens.

SAFETY AND TOLERABILITY

General (Tables 3[10,13–16,19,20,28,57,58,61–64] and 4[10,12–15,19,21,28,57,58,61–64]). Doxycycline is generally well tolerated with GI complaints and photosensitivity among the most common adverse events (AEs). Most available information on AEs in doxycycline are from studies examining doxycycline at prophylactic doses (100 mg/day), and there is limited information on AEs in doxycycline at treatment doses. At prophylactic doses of doxycycline, reports of mild and moderate AEs vary widely among studies from 0.6% to 84% of the study population.[13,20,21,59–61] This variation can be

FOI 26-3132 - Document 5

TABLE 3
Mild to moderate adverse events (AEs) associated with doxycycline prophylaxis and treatment*

Study location (design)	Sample size, dose, duration	Most frequent AE	Comparator	AE risk with doxycyline
Prophylaxis				
Kenya (RDBPC, semi-immune)[10]	55, 100 mg doxycycline hyclate daily, 10 weeks	Events: Abdominal pain 49 Diarrhea 9 Vomiting 6 Vaginitis 5	Azithromycin daily Azithromycin weekly Placebo	No difference between doxycycline and comparators
Thailand (RDB, non-immune)[62]	119, 100 mg doxycycline daily, 5 weeks	Nausea 16.7% Headache 10.7% Skin rash 10.7% Dizziness 6.3% Vomiting 4.4%	MQ	No difference between doxycycline and MQ
Africa (observational, non-immune)[63]	188, 100 mg doxycycline daily, 4 days	Nausea 33.0% Abdominal pain 22.9% Drowsiness 23.9% Headache 19.7% Vomiting 8.5% Dizziness 7.4% Rash 0.5%	MQ	Doxycycline group had significantly more nausea, abdominal pain, vomiting, drowsiness, headache, and dizziness than MQ goup.
Irian Jaya, Indonesia (RDBPC, non-immune)[13]	67, 100 mg doxycycline daily, 13 weeks	Skin-related 33% Cough 31% GI symptoms 24% Headache 16% Dizziness 9% Insomnia 6%	MQ Placebo	Doxycycline group had significantly fewer AE relative to MQ and placebo.
Thai-Burmese border (RPC, open-label, semi-immune, children aged 10–16 years)[14]	67, > 40 kg 100 mg doxycycline daily, < 40 kg 50 mg doxycycline daily, 5 months	Abdominal pain 16% Nausea 12% Diarrhea 8% Vomiting 4% Headache 34.3% Dizziness 26.8%	Doxycycline half-dose	Full-dose doxycycline more likely to have GI symptoms than half dose. Full-dose doxycyline less likely to have headache and dizziness than placebo.
Thai-Burmese border (R, open-label, semi-immune, children aged 10–16 years)[15]	95, > 40 kg 100 mg doxycycline daily, < 40 kg 50 mg doxycycline daily, 9 weeks	Dizziness 27% Nausea 20% Headache 17% Fever 15% Abdominal pain 8% Vomiting 7% Diarrhea 7%	CQ	No difference between doxycycline and CQ
Papua New Guinea (open-label, non-immune)[16]	55, 100 mg doxycycline daily, 3 weeks	Gastrointestinal 24% Photosensitivity 7.3%	Doxycycline + PQ	No difference between doxycycline and doxycycline + PQ
Somalia (observational, non-immune)[19]	52, 100 mg doxycycline daily, 7 weeks	Gastrointestinal 34.6% Photosensitivity 21.2% Lightheadedness 19.2%	MQ	Doxycycline had significantly higher rates of photosensitivity and gastrointestinal events than MQ.
Rwanda (observational, non-immune)[20]	28, 100 mg doxycycline daily, 2 months	Abdominal pain 33.3% Fatigue 23.8%	MQ	Doxycyline had higher rates of AE compared with MQ.
Somalia (observational, non-immune)[57]	499, 100 mg doxycycline daily, varied time periods	Gastrointestinal 17% Dizziness 10% Insomnia or nightmares 9% Sunburn 5%	MQ	No significant difference between doxycycline and MQ, except MQ had more insomnia or nightmares.
Sub-Saharan Africa (RDB, placebo run-in phase, non-immune)[61]	153, 100 mg doxycycline	Neuropsychological 24% Gastrointestinal 14% Skin itching, reddening 5% Skin and vaginal itching, abnormal discharge 9%	MQ CP Atovaquone and Proguanil	Doxycycline had less neuropsychological events than mefloquine, and less adverse skin events than CQ + proguanil. No increased risk of vaginal candidiases with doxycycline.
Chad and Gabon (randomized groups, non-immune)[58]	275, 100 mg doxycycline monohydrate daily, 5 months	Events: Diarrhea 52 Epigastralgia 46 Nausea 30 Vertigo 14 Mouth ulcers 11 Pruritis 11 Skin rash 9 Sun sensitization 4	CP	Doxycycline had significantly less diarrhea, epigastralgia, mouth ulcers, urticaria, pruritis, rash, and sun sensitization than CP. No statistical difference in nausea and vertigo.
Varied countries (observational, non-immune)[64]	228, 100 mg doxycycline daily, at least 6 months	Gastrointestinal 35% Skin and vaginal 34% Neuropsychologic 30%	MQ CQ	Doxycycline had a higher proportion of skin, vaginal, and gastrointestinal events than MQ and CQ.
Treatment				
Brazil (R, open-label, semi-immune)[28]	31, 100 mg doxycycline twice a day for 5 days + 500 mg quinine every 8 hours for 3 days	Upper abdominal pain 41.4% Nausea 34.5% Vomiting 27.6% Asthenia 27.6% Headache 27.6%	AL	Frequency of AE were similar for both doxycycline and AL.

* MQ = mefloquine; GI = gastrointestinal; CQ = chloroquine; PQ = primaquine; CP = chloroquine-proguanil; AL = artemether/lumefantrine.

FOI 26-3132 - Document 5

TABLE 4
Severe adverse events (AE) associated with doxycycline prophylaxis and treatment

Study location (design)	Sample size, dose, duration	Severe AE	Discontinued because of AE
Prophylaxis			
Kenya (RDBPC, semi-immune)[10]	55, 100 mg doxycycline hyclate daily, 10 weeks	Recurrent vaginitis 1	1 withdrew
Thailand (RDB, non-immune)[62]	119, 100 mg doxycycline daily, 5 weeks	None	None
Gabon and Central African Republic (non-immune)[12]	171, 100 mg doxycyline hyclate daily, 4 months	Gastrointestinal side effects 11	11 withdrew
Africa (observational, non-immune)[63]	188, 100 mg doxycycline daily, 4 days	Esophagitis 1	1 discontinued, requiring further evaluation and treatment
Irian Jaya, Indonesia (RDBPC, non-immune)[13]	67, 100 mg doxycycline daily, 13 weeks	None	None
Thai-Burmese border (semi-immune, children aged 10–16 years)[14]	67, 100 mg doxycycline daily, 5 months	None	None
Thai-Burmese border (R, open-label, semi-immune, children aged 10–16 years)[15]	95, > 40 kg 100 mg doxycycline daily, < 40 kg 50 mg doxycycline daily, 9 weeks	None	None
Rwanda (observational, non-immune)[19]	28, 100 mg doxycycline daily, 2 months	Abdominal pain 1	1 discontinued
Somalia, Cambodia (observational, non-immune)[21]	900, 100 mg daily, 4 months, all received primaquine eradication course after travel	Sun sensitization 13 Gastroinestinal problems 2	15 discontinued
Somalia (Observational, non-immune)[57]	499, 100 mg doxycycline daily, varied time periods	Odynophagia 4	4 discontinued, required hospitalization because of dehydration
sub-Saharan Africa (RDB, placebo run-in phase, non-immune)[61]	153, 100 mg monohydrate daily, 7–9 weeks	Skin and vaginal itching, abnormal discharge 4 Skin itching, reddening 3 Gastrointestinal 3 Neuropsychological 1	5 withdrew, did not specify which AE
Chad and Gabon (randomized groups, non-immune)[58]	275, 100 mg doxycycline monohydrate daily, 5 months	Gastrointestinal effects 9 Headache 5 Sun sensitization 1	15 withdrew
Varied countries (observational, non-immune)[64]	228, 100 mg doxycycline daily, at least 6 months	Gastrointestinal 11 Skin and vaginal 9 Neuropsychologic 5	Did not specify. Regimen changes caused by AE was lower among those taking doxycycline than those taking CQ or MQ.
Treatment			
Brazil (R, open-lab,[28] semi-immune)[61]	31, 100 mg doxycycline twice a day for 5 days + 500 mg quinine every 8 hours for 3 days	Vomiting 1	1 withdrew

explained in part because of a high background rate of non-specific symptoms experienced by both non-immune travelers and semi-immune study subjects in malaria endemic areas, differing definitions of mild and moderate AEs, use of different salts and formulations of doxycycline, and different study populations.

Reports of AEs of doxycycline at treatment doses (100 mg twice a day) are very limited because studies often examine malaria treatment with doxycycline in combination with another drug such as quinine, and it is difficult to determine if symptoms are related to doxycycline alone, malaria, the accompanying drug, or because of a drug-drug interaction. One randomized, open-label study comparing treatment of malaria with doxycycline and quinine versus artemether-lumefantrine found that at least one AE was reported in 93.5% of patients treated with quinine-doxycycline and 89.2% of those treated with artemether-lumefantrine; statistically similar proportions.[28] No serious AEs were noted in both groups.[28]

Gastrointestinal adverse events. Gastrointestinal symptoms are usually mild to moderate and include nausea (4–33%) and abdominal pain (12–33%).[13–15,19,20,58,62,63] Nausea has been noted to be more common when doxycycline is ingested without food.[13,21] Vomiting (4–8%) and diarrhea (6–7.5%) are less commonly reported.[13–15,62,63] The GI symptoms requiring a change in medication have been reported infrequently (0.2–6.4%).[12,20,21]

Doxycycline and other tetracyclines are believed to be the most common cause of drug-induced esophageal disorders.[64,65] It is thought that long transit times of the pills in the esophagus contributes to mucosal inflammation and that ingestion of a sufficient amount of fluid, taking the pill while in an upright position, and avoiding taking the pill before bedtime could decrease the transit time and prevent inflammation.[65,66] Doxycycline has been implicated in esophagitis; among United States military personnel returning from Somalia, 4 of 499 (0.8%) who took daily doxycycline developed odynophagia with dehydration requiring hospitalization.[57] One other study, a shipboard survey, found esophagitis in 1 of 188 persons taking doxycyline prophylaxis.[63]

The effect of various doxycycline salts and formulations such as oral suspensions, tablets, capsules, and enteric-coated tablets are likely quite significant in their ability to cause GI adverse events. Gelatin capsules have been observed to be more sticky than tablets, delaying transit time or even adhering to the esophageal lining, and this is affected by the amount of fluids ingested with the pills.[65,67] Enteric-coated formulations are available

FOI 26-3132 - Document 5

and may decrease GI side effects. One double-blind, placebo-controlled, multiple-dose cross-over study compared the incidence of GI side effects of enteric-coated doxycycline hyclate pellets in capsules (Doryx, Parke-Davis, Morris-Plains, NJ) and doxycycline hyclate powder in capsules (Vibramycin, Pfizer, New York), the enteric-coated capsule formulation produced statistically significantly fewer episodes of nausea, vomiting, abdominal discomfort, and decreased appetite than did the non-enteric-coated capsules.[68] Differences in GI adverse events between doxycycline salts may exist. In the majority of studies in which doxycycline has been used for malaria prophylaxis, the source of doxycycline, the salts used, and the formulation was not reported. Because the exact active drug ingredient and the formulation used seems to affect the incidence and severity of GI adverse events, rates between studies are difficult to compare. However, one study[12] examining efficacy of doxycycline hyclate in the French Army noted a 6% withdrawal rate caused by GI AEs and attributed this to the hyclate salt acidity and capsule form. Fewer withdrawals caused by GI AEs (1.5% and 4.9% at two study sites) were observed in a follow-up study[58] looking at tolerability of doxycycline monohydrate. Although data are limited, it appears that enteric-coated hyclate formulations and monohydrate salts are better tolerated in terms of GI AEs than standard hyclate capsules or tablets.

Photosensitivity. An erythematous rash in sun-exposed areas has been reported to occur in 7.3–21.2% of persons[16,19,57] taking doxycycline for malaria prophylaxis. One study examined the tolerability of various malaria prophylactic regimens and found that doxycycline did not cause a significantly higher percentage of all skin events (photosensitivity not specified) when compared with other antimalarials. The rash resolves upon discontinuation of the drug. A study of 106 acne patients taking daily doxycycline found a high incidence (35.8%) of light-sensitive rash and suggested that the phenomenon is dose related; 20% of patients taking 150 mg/day of doxycycline developed a light-sensitive rash, whereas 42% of those taking 200 mg/day were affected.[69] It is suggested that patients with lighter complexions (Fitzpatrick skin types 1 and 2) may be more susceptible to doxycycline photosensitivity than patients with darker skin pigmentation.[70] Both ultraviolet A (UV-A) and ultraviolet B (UV-B) radiation have been implicated.[71] Although the mechanism of the phototoxic reaction has not been fully elucidated, it is believed to be mediated by excited-state singlet oxygen and free radicals after irradiation with UV-A radiation, thereby causing selective injury to mitochondria, within which doxycycline and other tetracyclines are localized.[72] Patients developing photosensitivity should be advised to stop taking doxycycline. Avoiding sun exposure, wearing protective clothing, and using a broad spectrum (protection against both UV-A and UV-B radiation) sunscreen can lower the risk of photosensitivity.[73,74]

Vulvovaginitis. Antibiotics such as tetracyclines are believed to suppress vaginal bacterial flora, resulting in overgrowth of candida.[75] A recent *in vitro* study found that tetracycline may also enhance *Candida albicans* virulence factors, but the authors note that it is still unclear as to what degree this contributes to clinical vaginitis.[76] Increased colonization with *C. albicans* after taking tetracyclines has been shown.[75,77,78] After a 3-week course of tetracycline, one study observed that the percentage of women with vaginal cultures positive for *C. albicans* increased from 7% to 24%.[77] Another study had similar findings for both tetracycline and minocycline.[78] Despite this increase in yeast colonization, both studies found that only a small percentage of women with positive candidal vaginal cultures had symptoms of vulvovaginitis.[77,78] In fact, other studies have found that only a small percentage of women develop clinical vulvovaginitis after taking tetracyclines.[10,79] In women taking tetracycline for acne, symptomatic vaginitis was observed in 5%.[79] In another study, only one of 21 (4.8%) women on daily doxycycline withdrew from the study because of repeated episodes of vaginitis.[10] Another study did not find an increased risk for vaginal candidiasis with doxcycyline.[61] Of note, reporting of vaginitis as an adverse drug reaction specifically to doxycycline when taken as malaria prophylaxis is limited because vaginitis is often included under the category of non-specific skin reactions and many of the early studies were done in male military personnel. Women taking doxycycline who have a predisposition to candidal vulvovaginitis, such as those with diabetes mellitus, past history of recurring vulvovaginitis, and those taking oral contraceptives, should consider carrying a self-treatment course of antifungals.

Other AEs. There are other AEs that have been reported in association with tetracyclines. These include case reports of benign intracranial hypertension with tetracycline, minocycline, and doxycycline, but this has been observed to resolve upon discontinuation of the medication and has not been described in someone taking doxycycyline for malaria prevention.[80–82] Skin hyper-pigmentation has been reported in the dermatological literature associated primarily with minocycline, and seems to mainly occur with long-term use and usually fades slowly after discontinuing treatment.[83–88] Only one case report mentions skin hyper-pigmentation associated with doxycycline in a patient who had taken 1 g dose of doxycycline daily for 12 years.[89] Other AEs mentioned in case reports associated with tetracyclines include postinflammatory elastolysis and tooth discoloration in an adult with long-term use (12 years).[90,91] Central nervous system symptoms such as vertigo and ataxia have been more frequently associated with minocycline as compared with doxycycline because of minocycline's greater lipid solubility.[92] *Clostridium difficile*-associated diarrhea (CDAD) has been reported in three patients taking doxycycline for malaria prophylaxis for between 2 and 6 weeks.[93] A more recent case-control study examining antibiotic use and subsequent CDAD found doxycycline to be protective, however, this study did not examine the duration and dose of antibiotic therapy.[94]

Comparative tolerability. There are varying reports of the comparative tolerability of doxycycline. Several non-randomized survey-based studies in military personnel have found a higher occurrence of photosensitivity, GI symptoms, headache, and light-headedness with doxycycline as compared with mefloquine, whereas others have found that doxycycline is better tolerated than mefloquine.[19,20,57,63,64] The type of doxycycline salt and formulation used in these studies were rarely reported, making it difficult to distinguish if the comparative tolerability of doxycycline to other antimalarials was affected by the type of doxycycline preparation used. One randomized, open-label trial found that while more participants taking doxycycline monohydrate withdrew from the study, their rates of adherence were higher compared with those taking chloroquine/progruanil.[58] However, another randomized, open-label trial found doxycycline (unspecified salt) to be tolerated as well as chloroquine.[15]

FOI 26-3132 - Document 5

The strongest evidence for doxycycline's relatively good tolerability is found in three randomized, double-blinded trials. Two of the studies found little or no difference in the frequency of AEs between doxycycline (unspecified salt) and mefloquine, and all AEs were mild and transient.[13,62] In the third study, doxycycline monohydrate, atovaquone/proguanil, mefloquine, and chloroquine plus proguanil were compared ($N = 623$ evaluable persons).[61] Severity of AEs was graded on a scale of 1–4 with 1 being trivial and 4 requiring hospitalization. Although no significant differences were seen between groups for mild (grade 1) or severe (grade ≥ 3) AEs, moderate AEs (grade = 2) differed significantly between groups. Moderate AEs were reported by 45% of chloroquine/proguanil users, 42% of mefloquine users, 33% of doxycycline monohydrate users, and 32% of atovaquone/proguanil users. Although not statistically significant, doxycycline monohydrate had the lowest incidence of severe adverse events (6%) of all regimens. Of these severe events, one-third was skin reactions. There were no significant differences in the rates of skin reaction or GI events between any of the regimens. When compared with the other medications, doxycycline monohydrate had an intermediate proportion of withdrawals, but this proportion did not differ significantly between the treatment arms. The authors concluded that of all four regimens, doxycycline monohydrate was one of the best tolerated.[61] Overall, the evidence suggests that doxycycline is tolerated as well as other malaria prophylactic regimens.

CONTRAINDICATIONS

Doxycycline is contraindicated in anyone with a history of hypersensitivity to any of the tetracyclines.

Duration of Use. There are very limited studies on the use of doxycycline for malaria prophylaxis for a prolonged period of time. The current FDA product insert approves use for up to 4 months.[95] One study surveyed 600 military personnel in Cambodia who took prophylactic doses of doxycycline for 12 months and 900 men deployed to Somalia for 4 months.[21] Doxycycline was well tolerated; only 7 (0.6%) in Cambodia and 15 (1.7%) in Somalia discontinued medication because of adverse events related to GI problems and photosensitivity. Another study surveyed 228 U.S. Peace Corps volunteers who had taken doxycycline prophylaxis for an average of 19 months.[64] Of these, 45 (20%) changed medications caused by adverse events such as GI symptoms, pruritic skin reactions, photosensitivity, and vaginal yeast infection. Compared with mefloquine in which 28% of respondents changed medication caused by adverse events, doxycycline was better tolerated. Of note, in these two studies, the types of adverse events reported were the same as those in persons using doxycycline for shorter periods of time. However, both studies used post-trip surveys and did not have information on adherence for all participants.

Although long-term safety and tolerability studies have not been performed with doxycycline as a malaria chemoprophylaxis, similar drugs in the same class, tetracycline and minocycline, have been used in equivalent doses (less than 1 g/day for tetracycline, 50 mg/day to 100 mg twice a day for minocycline) for a few months to several years in the treatment of various dermatological conditions. This suggests that doxycycline can be used for long-term malaria chemoprophylaxis. When used at this dosage, tetracyclines were found in some studies to cause no abnormalities in renal and hepatic function when taken for as long as 5 years and in one study, for as long as 13 years.[96–98] There have, however, been several case reports of minocycline-associated autoimmune hepatitis and systemic lupus erythematosus-like syndrome in persons who used minocycline at 100 mg or more daily for more than 1 year.[99–103] Of note, doxycycline users were not found to have an increased risk of hepatotoxicity in one matched case-control study.[104] Scleral, dental, and dermal hyperpigmentation has also been reported in patients taking long-term minocycline as early as 1 month after initiation of treatment,[86,87,105,106] but this hyperpigmentation has been shown to fade after discontinuation of the medication.[86,87,105,106] In a 2-year, double-blind comparison of minocycline (100 mg twice a day) and hydroxychloroquine for the treatment of rheumatoid arthritis, only 10% of patients withdrew because of rash or GI symptoms.[106] Overall, these case reports and studies observed rare to occasional adverse events associated with long-term use of other tetracyclines.

THERAPEUTIC INDEX AND OVERDOSE

Tetracycline antibiotics are relatively non-toxic. Exceeding the recommended dosage of doxycycline may result in increased incidence of ADRs. There has been one case report of an individual who took 1 g of doxycycline/day for 12 years.[89] He was found to have hepatocellular necrosis with cholestasis, nephrotoxicity, leucopenia with anemia, skin hyperpigmentaion, and intermittent supraventricular tachycardia with sporadic Wenckebach heart block. He was not found to have any of the common doxycycline side effects such as GI symptoms or photosensitivity. After discontinuation of the drug, most of these symptoms resolved with the exception of residual cutaneous hyperpigmentation, relapsing cardiac arrythmias, and increased bilirubin.[89] In the case of overdosage, the medication should be discontinued and supportive measures and symptomatic treatment instituted.[95]

DRUG INTERACTIONS

Doxycycline was introduced into clinical practice long before the recognition of drug-drug interactions and without currently required pre-licensure *in vitro* or *in vivo* studies.[107] The potential for drug-drug interactions with doxycycline based on a full panel of *in vitro* studies with phase I metabolizing enzymes (cytochrome P450), phase II enzymes, and transporter mediated drug interactions has not been reported. On the basis of a single *in vitro* study showing that doxycycline inhibits the metabolism of quinine to 3-hydroxyquinine, the major metabolic pathway known to be enzymatically catalyzed by CYP 3A4, doxycycline is thought to be a modest inhibitor of CYP 3A4. Because CYP 3A4 is the most common phase 1 enzyme involved in drug metabolism, the spectrum of potential drug-drug interactions is wide and is not well defined. Furthermore, *in vitro* interactions do not always predict clinically significant effects. Information on drug-drug interactions with doxycycline is based on clinical observations and predictions are based on limited data accumulated over the last 40 years of clinical use, as described below.

Tetracyclines can chelate divalent and trivalent cations in certain medications that may cause a decreased absorption of the tetracycline and the medication when given concurrently.[3] These medications include antacids with aluminum, calcium

FOI 26-3132 - Document 5

or magnesium, laxatives with magnesium, and oral iron preparations. Other medications decreasing absorption of tetracyclines include antidiarrheal agents containing kaolin, pectin, or bismuth subsalicylate. Medications such as these should be taken a few hours before doxycycline.[3,108]

Although a possible interaction with oral contraceptives (OCs) has been cited in older literature,[3] current evidence has not supported this information.[109–111] The overall OC failure rate in women who were also treated with antibiotics was found to be 1.2–1.6%, and this failure is thought to occur in individuals in whom plasma concentrations of ethinyl estradiol are decreased.[110] Furthermore, the failure rate observed in concurrent administration of OC and antibiotics is well within the 1–3% failure rate observed with typical OC use.[110,112] Therefore, recent evidence suggests that doxycycline can be used concurrently with OCs without leading to a higher rate of contraceptive failure than would be expected among OC users not currently taking antibiotics.

Tetracyclines may potentiate the effect of oral anticoagulants by impairing the use of prothrombin and decreasing vitamin K production by intestinal bacteria.[3] The literature is limited, and there has been only one case report of a woman well controlled on warfarin, who developed an increased international normalized ratio and retroperitoneal bleeding when doxycycline was administered.[113] It was hypothesized that in addition to the aforementioned mechanism, competition with protein binding or the occurrence of an inhibitory mechanism by the cytochrome P450 system could be factors.[113] Patients on oral anticoagulants should have their prothrombin times monitored closely and dosage adjusted as needed.[3]

As a bacteriostatic drug, tetracyclines may interfere with the bactericidal action of aminoglycosides and penicillin[3,36] but this has only been reported *in vitro*,[114] and may not be of clinical significance. Doxycycline can be used concurrently with ciprofloxacin and azithromycin, two antibiotics commonly given to travelers for self-treatment of traveler's diarrhea.

Fatal nephrotoxicity has been reported in patients receiving methoxyflurane anesthesia and tetracycline.[115–117] Although this has not been observed with doxycycline, specifically, concurrent use of tetracyclines and methoxyflurane anesthesia is not recommended.

Concurrent use of digoxin and oral antibiotics such as tetracyclines may increase serum digoxin concentrations in some individuals. It is believed that the tetracycline alters gut flora, diminishing digoxin conversion to inactive metabolites, resulting in increased serum digoxin concentrations.[118,119] However, the data is limited and there have been no reports of toxicity caused by concurrent administration of digoxin and tetracyclines.

Barbiturates, carbamazepine, and phenytoin are believed to decrease the half-life of doxycycline by inducing microsomal enzyme activity.[41,120] It has been suggested to double the doxycycline dosage (take twice daily) in those taking antiepileptics.[120]

Theoretically, antibiotics such as doxycycline could decrease the effectiveness of the oral typhoid vaccine Ty21a by preventing replication of the live attenuated *Salmonella* Ty21a bacteria used in the vaccine. However, there is no experimental data in animals or humans to support this position. Nevertheless, most experts advise not giving doxycycline within 24 hours of the oral typhoid vaccine.[1] Because the immunization schedule for oral typhoid vaccine is recommended to be completed at least 1 week before potential exposure to *Salmonella typhi*,[121] there should be no opportunity for interaction between the oral typhoid vaccine and doxycycline in most travelers.

There are no known drug interactions between doxycycline and antiretrovirals.

SPECIAL POPULATIONS

Children. Doxcycycline is not recommended for children < 8 years of age because tetracyclines have been shown to cause permanent discoloration of the teeth.[122,123] Tetracyclines form a complex with calcium orthophosphate that becomes incorporated into bones and teeth undergoing calcification.[124] Tooth discoloration is permanent because remodeling and calcium exchange do not occur after calcification is completed. Some believe teeth staining is related to the total dose of tetracycline[122,123] while others argue that it is caused by repeated courses.[125,126] It is also thought that timing of tetracycline administration during tooth development, especially during early dentin formation, determines the degree of discoloration.[126] By 8 years of age the dentin and calcium-rich enamel has formed on almost all teeth (except wisdom teeth), and therefore doxycycline can be taken after this age. There are some who recommend that the lower age limit for taking doxycycline should be 12, when most children have had their primary teeth replaced by their adult dentition. Doxycycline, however, may cause less tooth discoloration than other tetracyclines because it has a lower binding affinity to calcium.[127,128] In fact, one study found that a short course of doxycycline given to children for Rocky Mountain spotted fever did not cause clinically significant staining of teeth.[129]

Of note, doxycycline is considered the drug of choice for treatment of infections in which the risks of tooth discoloration is outweighed by the benefit of disease treatment, such as presumed or confirmed rickettsial infection, ehrlichiosis, murine typhus, or inhalational anthrax.[129–135] Because there are other drug alternatives for treatment and prophylaxis of malaria in children < 8 years of age, use of doxycycline is not recommended.

The formation of the tetracycline and calcium orthophosphate complex in developing bone is thought to cause growth retardation, but the evidence is limited. Decreased long bone growth rate has been observed in premature infants given tetracycline but is reversible upon discontinuation of the drug.[136,137] There are no studies showing tetracycline-induced growth retardation in term infants or older children.

Pregnant women. Doxycycline is a category D drug; therefore, the possibility of pregnancy in a woman of childbearing age must be addressed before administration of this drug.[124] Doxycycline is not recommended for malaria prevention or treatment in pregnancy, except for the treatment of life-threatening multidrug-resistant *P. falciparum* when no other treatment options are available. No congenital malformations have been associated with its use,[124,138,139] and some limited evidence suggests that use of doxycycline in the first trimester might not cause adverse events in the fetus.[140,141] However, tetracyclines cross the placenta and have been observed to cause discoloration of teeth when given during the second or third trimester of pregnancy.[142,143] Because deciduous teeth begin to calcify at 5–6 months gestation, use of tetracycline during this time can result in staining.[124] Additionally, on the basis of

FOI 26-3132 - Document 5

observed retardation of bone growth in premature infants,[137] there are concerns of inhibition of bone growth in the fetus.

Tetracyclines have also been associated with fatal liver necrosis in pregnancy.[138,144,145] These reports, however, are rare and have usually involved higher doses of doxycycline than those used for malaria prophylaxis and treatment.[146]

For women planning on becoming pregnant, there is no need to wait a specific period of time after doxycycline use before attempting to conceive, based on the short half-life (15–25 hours) of the drug.[3,36] If women or their health care providers wish to decrease the amount of doxycycline in the body before conception, after 6 half-lives (3–6 days) only 2% of the doxycycline remains in the body.[147]

Breastfeeding. Data are extremely limited on the use of doxycycline in breastfeeding women. Doxycycline is excreted in breast milk at concentrations 30–40% of that found in maternal blood,[138,148] and it is thought that the drug's absorption by infants is inhibited by the calcium in the breast milk. Furthermore, tetracycline was undetectable in breastfed infants whose mothers were taking tetracycline.[149] Tetracycline has also been assessed by the American Academy of Pediatrics to be compatible with breastfeeding.[149] Although data for doxycycline and breastfeeding are limited, most experts feel that the theoretical possibility of teeth discoloration and bone growth inhibition in the nursing baby would be remote. Concerned mothers or doctors may consider alternative drugs such as mefloquine, or for mothers nursing babies weighing more than 5 kg, atovaquone/proguanil.

Medical conditions. In patients with impaired renal or hepatic function, the dose of doxycycline does not have to be adjusted because it is excreted as an inactive chelated product by a complex process of back diffusion in the small intestine.[150] Additionally, the clearance of doxycycline has been shown to be similar to those with normal renal function and in those with renal failure.[151]

In patients with seizure disorders in whom mefloquine is contraindicated, doxycycline can be given as an alternative.[147] However, antiepileptics have been noted to shorten the half-life of doxycycline and dosing adjustments must be made accordingly (see Drug Interactions section).

Doxycyline (Vibramycin) syrup contains metabisulfite, which can cause allergic reactions, including anaphylaxis and asthmatic episodes in susceptible individuals.[3]

Persons being treated for skin conditions with minocycline and who are in need of malaria prophylaxis should stop minocycline and start doxycycline. There is more data on the antimalarial efficacy of doxycycline compared with minocycline, therefore minocycline is not recommended as a replacement for doxycycline. Additionally, there have been case reports of autoimmune hepatitis in persons taking 100 mg or more of minocycline daily, whereas doxycycline has been observed to have less hepatotoxicity than other tetracyclines and less adverse events than minocycline.[100,101,104,152] After completion of doxycycline for malaria prophylaxis, minocycline can be resumed.

COST

The drug cost for doxycycline varies by doxycycline salt, enteric formulation, or brand name versus generic. One article examining the cost considerations of malaria chemoprophylaxis looked at the average wholesale price (prices from 2006) of various antimalarial medications and found that generic doxycycline hyclate was much cheaper (100 mg, US $0.25 per dose) than generic doxycycline monohydrate (100 mg, $2.13 per dose) and that generic doxycycline monohydrate was much cheaper than the brand name (Vibratabs, doxycycline monohydrate 100 mg, $4.51 a dose).[153] In this same work, the direct costs of various malaria chemoprophylaxis regimens were compared. For a 14-day stay, including pre- and post-exposure doses, the drug cost for prophylaxis was $11.00 for generic doxycycline hyclate compared with $84.64 for mefloquine and $113.39 for atovaquone/proguanil.[153] Using generic doxycycline monohydrate brings up the cost of prophylaxis to that of mefloquine, and using brand name doxycycline monohydrate brings the costs up even further. To look at more recent costs, prices of antimalarials were examined online for the average wholesale price. A full course of malaria prophylaxis for a 14-day stay in a malaria-endemic area costs $18.49 for generic doxycycline hyclate capsules, $65.79 for generic doxycycline monohydrate capsules, $122.50 for Vibratabs, $406.35 for Doryx (enteric-coated doxycycline hyclate), $80.48 for mefloquine, and $159.94 for atovaquone/proguanil.[154]

EXCHANGING DOXYCYCLINE FOR OTHER ANTIMALARIALS

There is very limited information on the best regimen for changing from doxycycline to other antimalarials, and recommendations for doing so are based on considering several issues such as drug-drug interactions, half-life differences, and differences in mechanism of action. If changing between atovaquone/proguanil and doxycycline, note that the current atovaquone-proguanil label lists a pharmacokinetic interaction of "concomitant treatment (of *P. falciparum* malaria) with tetracycline (with atovaquone) has been associated with approximately a 40% reduction in plasma concentrations of atovaquone,"[155] however, there are no accessible published studies that support this. On the contrary, early *in vitro* studies of atovaquone plus doxycycline and atovaquone plus tetracycline showed potentiation between the two drugs, which was later confirmed in clinical studies by improved cure rates when compared with atovaquone alone.[156–158] Therefore, if changing from doxcycyline to atovaquone/proguanil for malaria treatment or prophylaxis, doxycycline can be stopped and atovaquone/proguanil started at the same time. However, the recommended length of the post-trip course of prophylactic medications may change because the drugs target different stages of the *Plasmodium* life cycle. The Centers for Disease Control and Prevention (CDC) currently recommends that the atovaquone/proguanil is continued for up to 4 weeks after leaving the endemic area depending on when the change was made.[1] If changing from atovaquone/proguanil to doxycycline at any time, a full prophylactic course of doxycycline should be taken (for the duration of the stay and 4 weeks after departure). Changing from doxycycline to other antimalarials such as chloroquine or mefloquine will be impacted by the difference in half-lives of these medications. Doxycycline's half-life of 15–25 hours is much shorter than that of chloroquine (6–60 days) and mefloquine (2–3 weeks).[1] A change from doxycycline to chloroquine or mefloquine before travel would still require that chloroquine and mefloquine are taken 1 and 2 weeks before exposure, respectively, to attain effective blood

FOI 26-3132 - Document 5

concentrations before travel. It is not recommended to change from doxycycline to chloroquine or mefloquine during or after travel because effective blood concentrations will not be achieved for a few weeks, leaving the traveler unprotected during this time.

Received May 19, 2010. Accepted for publication January 25, 2011.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention, the U.S. Department of Defense, the Department of the Army, or the Walter Reed Army Institute.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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Received: 5 June 2020 | Accepted: 9 June 2020

DOI: 10.1111/dth.13810

FOI 26-3132 - Document 5

DERMATOLOGIC THERAPY WILEY

LETTER

Further aspects of doxycycline therapy in COVID-19

Dear Editor,

In their work, Conforti et al urge experts to broadly investigate the combination of doxycycline and hydroxychloroquine in treating coronavirus disease (COVID-19).[1] The tetracycline antibiotic doxycycline is a nontoxic inhibitor of mitochondrial biogenesis and cellular respiration[2] besides other known pleiotropic properties.[1] Now that the World Health Organization has just halted hydroxychloroquine trials for COVID-19 because of safety reasons, the time has come to find auxiliary compounds so as to give additional benefit to doxycycline.

We also need to take into account that quite a lot of similarities in metabolic pathways of virally infected and cancer cells have been observed.[3] Viruses usually target mitochondria as cellular power houses and various interplays have been detected between viruses and mitochondrial dynamics.[4] Most viruses require aerobic glycolysis as the energy source for replication and its inhibition could attenuate this process.[4]

Vitamin C is a broad-spectrum antiviral agent[5] and an inhibitor of aerobic glycolysis.[2] The combined administration of doxycycline with vitamin C resulted in a robust eradication of cancer stem cells (CSCs) in in vitro experiments by blocking mitochondrial protein translation and ATP production from glycolysis.[2] The addition of azithromycin further boosted CSC clearance.[2]

Sargiacomo et al also proposed the use of doxycycline or azithromycin in COVID-19.[6] Their concept assumes that SARS-CoV-2 prefers chronologically aged, senescent lung cells for binding and replication causing stormy inflammation and subsequent fibrosis, suggesting the application of senolytic drugs such as doxycycline to prevent fibrotic transformation.[6]

I believe that a combined approach regarding CSC elimination[2] may be transmissible into antiviral therapy. Supplementation of doxycycline with the amplifier vitamin C may result in mitochondrial damage of virally compromised cells, the attenuation of immune response by the inhibition of glycolysis in pro-inflammatory immune cells,[7] and a stronger suppression on postinflammatory fibrosis[8] than with doxycycline alone.

In vitro very low concentration of doxycycline is able to block mitochondria,[2] and this antiinflammatory dose is approved up to a 12-month use in rosacea[9] so a prolonged course together with vitamin C may also be an inexpensive, safe, and promising approach in antiviral prophylaxis and treatment.

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Zn^{2+} Inhibits Coronavirus and Arterivirus RNA Polymerase Activity *In Vitro* and Zinc Ionophores Block the Replication of These Viruses in Cell Culture

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Abstract

Increasing the intracellular Zn^{2+} concentration with zinc-ionophores like pyrithione (PT) can efficiently impair the replication of a variety of RNA viruses, including poliovirus and influenza virus. For some viruses this effect has been attributed to interference with viral polyprotein processing. In this study we demonstrate that the combination of Zn^{2+} and PT at low concentrations (2 μM Zn^{2+} and 2 μM PT) inhibits the replication of SARS-coronavirus (SARS-CoV) and equine arteritis virus (EAV) in cell culture. The RNA synthesis of these two distantly related nidoviruses is catalyzed by an RNA-dependent RNA polymerase (RdRp), which is the core enzyme of their multiprotein replication and transcription complex (RTC). Using an activity assay for RTCs isolated from cells infected with SARS-CoV or EAV—thus eliminating the need for PT to transport Zn^{2+} across the plasma membrane—we show that Zn^{2+} efficiently inhibits the RNA-synthesizing activity of the RTCs of both viruses. Enzymatic studies using recombinant RdRps (SARS-CoV nsp12 and EAV nsp9) purified from *E. coli* subsequently revealed that Zn^{2+} directly inhibited the *in vitro* activity of both nidovirus polymerases. More specifically, Zn^{2+} was found to block the initiation step of EAV RNA synthesis, whereas in the case of the SARS-CoV RdRp elongation was inhibited and template binding reduced. By chelating Zn^{2+} with MgEDTA, the inhibitory effect of the divalent cation could be reversed, which provides a novel experimental tool for *in vitro* studies of the molecular details of nidovirus replication and transcription.

Citation: te Velthuis AJW, van den Worm SHE, Sims AC, Baric RS, Snijder EJ, et al. (2010) Zn^{2+} Inhibits Coronavirus and Arterivirus RNA Polymerase Activity *In Vitro* and Zinc Ionophores Block the Replication of These Viruses in Cell Culture. PLoS Pathog 6(11): e1001176. doi:10.1371/journal.ppat.1001176

Editor: Raul Andino, University of California San Francisco, United States of America

Received May 17, 2010; **Accepted** October 1, 2010; **Published** November 4, 2010

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Funding: This work was supported by the Netherlands Organization for Scientific Research (NWO) with grants from the Council for Chemical Sciences (NWO-CW grant 700.55.002 and 700.57.301) and an NWO Toptalent grant (021.001.037). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Zinc ions are involved in many different cellular processes and have proven crucial for the proper folding and activity of various cellular enzymes and transcription factors. Zn^{2+} is probably an important cofactor for numerous viral proteins as well. Nevertheless, the intracellular concentration of free Zn^{2+} is maintained at a relatively low level by metallothioneins, likely due to the fact that Zn^{2+} can serve as intracellular second messenger and may trigger apoptosis or a decrease in protein synthesis at elevated concentrations [1,2,3]. Interestingly, in cell culture studies, high Zn^{2+} concentrations and the addition of compounds that stimulate cellular import of Zn^{2+}, such as hinokitol (HK), pyrrolidine dithiocarbamate (PDTC) and pyrithione (PT), were found to inhibit the replication of various RNA viruses, including influenza virus [4], respiratory syncytial virus [5] and several picornaviruses [6,7,8,9,10,11]. Although these previous studies provided limited mechanistic information, this suggests that intracellular Zn^{2+} levels affect a common step in the replicative cycle of these viruses.

In cell culture, PT stimulates Zn^{2+} uptake within minutes and inhibits RNA virus replication through a mechanism that has only been studied in reasonable detail for picornaviruses [11,12]. *In vitro* studies with purified rhinovirus and poliovirus 3C proteases revealed that protease activity was inhibited by Zn^{2+} [13,14], which is in line with the inhibition of polyprotein processing by zinc ions that was observed in cells infected with human rhinovirus and coxsackievirus B3 [11]. The replication of segmented negative-strand RNA viruses such as influenza virus, however, does not depend on polyprotein processing and the effect of PDTC-mediated Zn^{2+} import was therefore hypothesized to result from inhibition of the viral RNA-dependent RNA polymerase (RdRp) and cellular cofactors [4]. Moreover, an inhibitory effect of Zn^{2+} on the activity of purified RdRps from rhinoviruses and hepatitis C virus was noted, but not investigated in any detail [15,16].

Details on the effect of zinc ions are currently largely unknown for nidoviruses. This large group of positive-strand RNA (+RNA) viruses includes major pathogens of humans and livestock, such as severe acute respiratory syndrome coronavirus (SARS-CoV), other human coronaviruses, the arteriviruses equine arteritis virus (EAV), and porcine reproductive and respiratory syndrome virus (PRRSV) [17,18]. The common ancestry of nidoviruses is reflected in their similar genome organization and expression strategy, and in the conservation of a number of key enzymatic functions in their

Author Summary

Positive-stranded RNA (+RNA) viruses include many important pathogens. They have evolved a variety of replication strategies, but are unified in the fact that an RNA-dependent RNA polymerase (RdRp) functions as the core enzyme of their RNA-synthesizing machinery. The RdRp is commonly embedded in a membrane-associated replication complex that is assembled from viral RNA, and viral and host proteins. Given their crucial function in the viral replicative cycle, RdRps are key targets for antiviral research. Increased intracellular Zn^{2+} concentrations are known to efficiently impair replication of a number of RNA viruses, e.g. by interfering with correct proteolytic processing of viral polyproteins. Here, we not only show that corona- and arterivirus replication can be inhibited by increased Zn^{2+} levels, but also use both isolated replication complexes and purified recombinant RdRps to demonstrate that this effect may be based on direct inhibition of nidovirus RdRps. The combination of protocols described here will be valuable for future studies into the function of nidoviral enzyme complexes.

large replicase polyproteins [19]. A hallmark of the corona- and arterivirus replicative cycle is the transcription of a 5′- and 3′-coterminal nested set of subgenomic (sg) mRNAs from which the viral structural and accessory protein genes are expressed [20,21].

Analogous to picornaviruses [13,22], zinc ions were demonstrated to inhibit certain proteolytic cleavages in the processing of the coronavirus replicase polyproteins in infected cells and cell-free systems [23,24]. In this study we report that the zinc-ionophore pyrithione (PT) in combination with Zn^{2+} is a potent inhibitor of the replication of SARS-coronavirus (SARS-CoV) and equine arteritis virus (EAV) in cell culture. To assess whether - besides a possible effect on proteolytic processing - nidovirus RTC subunits and RNA synthesis are directly affected by Zn^{2+}, we employed *in vitro* systems for SARS-CoV and EAV RNA synthesis that are based on membrane-associated RTCs isolated from infected cells (from here on referred to as RTC assays) [25,26]. In addition, we used *in vitro* recombinant RdRp assays to directly study the effect of zinc ions on the RdRps of SARS-CoV and EAV [27,28].

Using these independent *in vitro* approaches, we were able to demonstrate that Zn^{2+} directly impairs nidovirus RNA synthesis, since it had a strong inhibitory effect in both RTC and RdRp assays. Interestingly, the Zn^{2+}-mediated inhibition could be reversed through the addition of a Zn^{2+} chelator (MgEDTA). We therefore applied this compound to stop and restart the *in vitro* RNA-synthesizing activity at will. This convenient tool allowed us to study various mechanistic aspects of arteri- and coronavirus RNA synthesis in more detail. Additionally, the zinc-mediated inhibition of nidovirus RNA synthesis described here may provide an interesting basis to further explore the use of zinc-ionophores in antiviral therapy.

Results

Zinc and pyrithione inhibit nidovirus replication *in vivo*

Zinc ions are involved in many different cellular processes, but the concentration of free Zn^{2+} is maintained at a relatively low level by metallothioneins [1]. Zn^{2+} and compounds that stimulate import of Zn^{2+} into cells, such as PT, were previously found to inhibit replication of several picornaviruses, including rhinoviruses, foot-and-mouth disease virus, coxsackievirus, and mengovirus in cell culture [6,7,8,9,10,11]. To determine whether Zn^{2+} has a similar effect on nidoviruses, we investigated the effect of PT and Zn^{2+} on the replication of EAV and SARS-CoV in Vero-E6 cells, using reporter viruses that express green fluorescent proteins (GFP), i.e., EAV-GFP [29] and SARS-CoV-GFP [30]. EAV-GFP encodes an N-terminal fusion of GFP to the viral nonstructural protein 2 (nsp2), one of the cleavage products of the replicase polyproteins, and thus provides a direct readout for translation of the replicase gene. In SARS-CoV-GFP, reporter expression occurs from sg mRNA 7, following the replacement of two accessory protein-coding genes (ORFs 7a and 7b) that are dispensable for replication in cell culture.

We first assessed the cytotoxicity of a range of PT concentrations (0–32 μM) in the presence of 0 to 8 μM $ZnOAc_2$. Treatment with PT of concentrations up to 32 μM in combination with <4 μM $ZnOAc_2$ did not reduce the viability of mock-infected cells after 18 h (Fig. 1A), as measured by the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) viability assay. As elevated Zn^{2+} concentrations are known to inhibit cellular translation, we also used metabolic labeling with ^{35}S-methionine to assess the effect of PT and Zn^{2+} on cellular protein synthesis. Incubation of Vero-E6 cells for 18 h with the combinations of PT and Zn^{2+} mentioned above, followed by a 2-h metabolic labeling, revealed no change in overall cellular protein synthesis when the concentration of $ZnOAc_2$ was <4 μM (data not shown).

Using these non-cytotoxic conditions we subsequently tested the effect of PT and $ZnOAc_2$ on EAV-GFP and SARS-CoV-GFP replication. To this end, Vero-E6 cells in 96-well plates were infected with a multiplicity of infection (m.o.i.) of 4. One hour post infection (h p.i.), between 0 and 32 μM of PT and 0, 1, or 2 μM $ZnOAc_2$ were added to the culture medium. At 17 h p.i., a time point at which GFP expression in untreated infected cells reaches its maximum for both viruses, cells were fixed, and GFP fluorescence was quantified.

The reporter gene expression of both SARS-CoV-GFP and EAV-GFP was already significantly inhibited in a dose-dependent manner by the addition of PT alone (Fig. 1B and C). This effect was significantly enhanced when 2 μM of Zn^{2+} was added to the medium. We found that addition of $ZnOAc_2$ alone also reduced virus replication, but only at levels that were close to the 50% cytotoxicity concentration (CC_{50}) of $ZnOAc_2$ in Vero-E6 cells (~70 μM, data not shown). This is likely due to the poor solubility of Zn^{2+} in phosphate-containing medium and the inefficient uptake of Zn^{2+} by cells in the absence of zinc-ionophores. The combination of 2 μM PT and 2 μM $ZnOAc_2$ resulted in a 98±1% and 85±3% reduction of the GFP signal for EAV-GFP and SARS-CoV-GFP, respectively. No cytotoxicity was observed for this combination of PT and $ZnOAc_2$ concentrations. From the dose-response curves in Fig. 1, a CC_{50} value of 82 μM was calculated for PT in the presence of 2 μM zinc. Half maximal inhibitory concentrations (IC_{50}) of 1.4 μM and 0.5 μM and selectivity indices of 59 and 164 were calculated for SARS-CoV and EAV, respectively.

Zn^{2+} reversibly inhibits the RNA-synthesizing activity of isolated nidovirus RTCs

We previously developed assays to study the *in vitro* RNA-synthesizing activity of RTCs isolated from cells infected with SARS-CoV or EAV [25,26]. In these RTC assays [α-^{32}P]CMP is incorporated into both genomic (replication) and sg mRNA (transcription) (Fig. 2). This allowed us to monitor the synthesis of the same viral RNA molecules that can be detected by hybridization of RNA from nidovirus-infected cells. A benefit of these assays is that the activity does not depend on continued protein synthesis and that it allows us to study viral RNA synthesis

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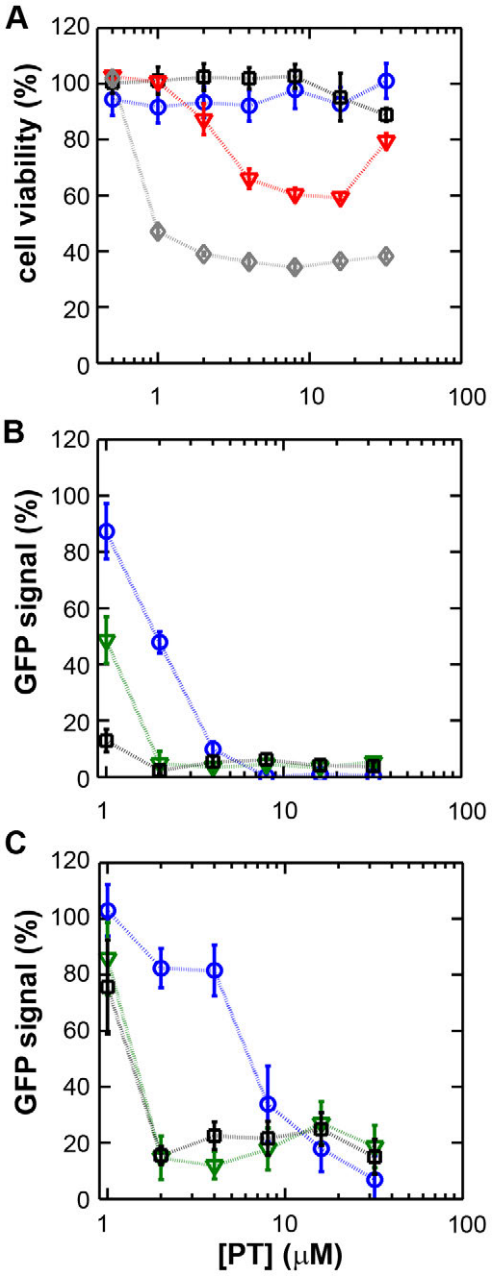


Figure 1. The zinc ionophore pyrithione inhibits nidovirus replication in cell culture. (**A**) Cytotoxicity of PT in Vero-E6 cells in the absence (blue circles) or presence of 2 (black squares), 4 (red triangles), or 8 μM (gray diamonds) $ZnOAc_2$ as determined by the MTS assay after 18 hours of exposure. (**B**) Dose-response curves showing the effect of PT and Zn^{2+} on the GFP fluorescence in Vero-E6 cells infected with a GFP-expressing EAV reporter strain at 17 h p.i. Data were normalized to GFP expression in infected, untreated control cultures (100%). The different Zn^{2+} concentrations added to the medium were 0 (blue circles), 1 (green triangles), or 2 μM $ZnOAc_2$ (black squares). (**C**) Effect of PT and Zn^{2+} on the GFP fluorescence in Vero-E6 cells infected with a GFP-expressing SARS-CoV reporter strain at 17 h p.i. Data were normalized to GFP expression in infected untreated control cells (100%). Colors for different Zn^{2+} concentrations as in Fig. 1B. Error bars indicate the standard deviation (n = 4).
doi:10.1371/journal.ppat.1001176.g001

independent of other aspects of the viral replicative cycle [26]. To investigate whether the inhibitory effect of PT and zinc ions on nidovirus replication in cell culture is reflected in a direct effect of

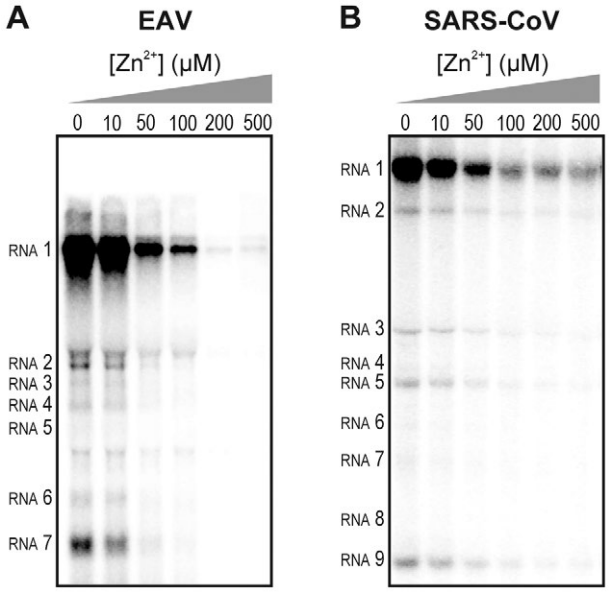


Figure 2. Inhibition of the *in vitro* RNA-synthesizing activity of isolated RTCs by Zn^{2+}. Incorporation of [α-^{32}P]CMP into viral RNA by EAV (**A**) and SARS-CoV (**B**) in RTC assays in the presence of various Zn^{2+} concentrations, as indicated above each lane.
doi:10.1371/journal.ppat.1001176.g002

Zn^{2+} on viral RNA synthesis, we tested the effect of Zn^{2+} addition on RTC activity. For both EAV (Fig. 2A) and SARS-CoV (Fig. 2B), a dose-dependent decrease in the amount of RNA synthesized was observed when $ZnOAc_2$ was present. For both viruses, a more than 50% reduction of overall RNA-synthesis was observed at a Zn^{2+} concentration of 50 μM, while less than 5% activity remained at a Zn^{2+} concentration of 500 μM. Both genome synthesis and sg mRNA production were equally affected.

To test whether the inhibition of RTC activity by Zn^{2+} was reversible, RTC reactions were started in the presence or absence of 500 μM Zn^{2+}. After 30 min, these reactions were split into two aliquots and magnesium-saturated EDTA (MgEDTA) was added to one of the tubes to a final concentration of 1 mM (Fig. 3A). We used MgEDTA as Zn^{2+} chelator in these *in vitro* assays, because it specifically chelates Zn^{2+} while releasing Mg^{2+}, due to the higher stability constant of the ZnEDTA complex. Uncomplexed EDTA inhibited RTC activity in all reactions (data not shown), most likely by chelating the Mg^{2+} that is crucial for RdRp activity [27,28], whereas MgEDTA had no effects on control reactions without Zn^{2+} (Fig. 3B, compare lane 1 and 2). As shown in Fig. 2, the EAV RTC activity that was inhibited by Zn^{2+} (Fig. 3B&C, lane 3) could be restored by the addition of MgEDTA (Fig. 3B, lane 4) to a level observed for control reactions without Zn^{2+} (Fig. 3B, lane 1). Compared to the untreated control, the EAV RTC assay produced approximately 30% less RNA, which was consistent with the 30% shorter reaction time after the addition of the MgEDTA (100 versus 70 min for lanes 1 and 4, respectively). Surprisingly, SARS-CoV RTC assays that were consecutively supplemented with Zn^{2+} and MgEDTA incorporated slightly more [α-^{32}P]CMP compared to untreated control reactions (Fig. 3C; compare lane 1 and 4). This effect was not due to chelation of the Zn^{2+} already present in the post-nuclear supernatant (PNS) of SARS-CoV-infected cells, as this increase was not observed when MgEDTA was added to a control reaction without additional Zn^{2+} (Fig. 3C, lane 2).

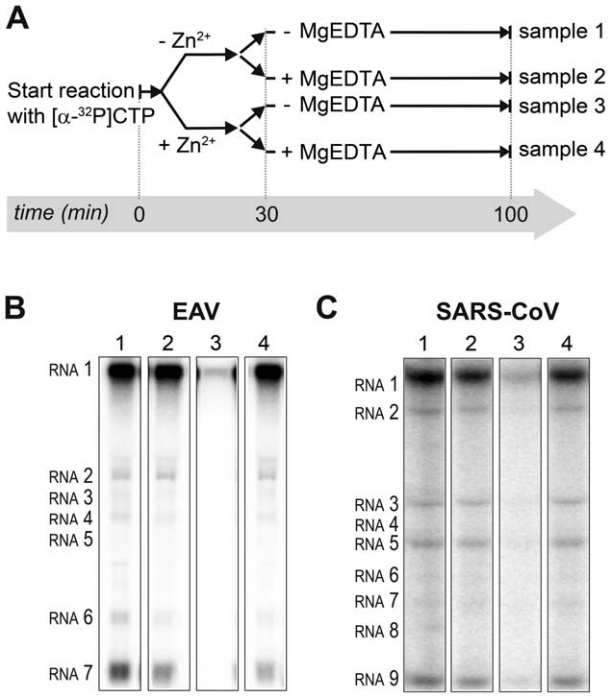


Figure 3. Inhibition of nidovirus RTC activity by Zn^{2+} can be reversed by chelation. (**A**) Schematic representation of the *in vitro* assays with isolated RTCs, which were initiated with [α-^{32}P]CTP, either in the absence (sample 1 and 2) or presence of 500 μM Zn^{2+}. After a 30-min incubation at 30°C, both the untreated and Zn^{2+}-treated samples were split into two aliquots, and 1 mM of the Zn^{2+} chelator MgEDTA was added to samples 2 and 4. All reactions were subsequently incubated for another 70 min before termination. (**B**) Analysis of RNA products synthesized in assays with EAV RTCs. Numbers above the lanes refer to the sample numbers described under (A). (**C**) *In vitro* activity assay with SARS-CoV RTCs.
doi:10.1371/journal.ppat.1001176.g003

Zinc ions affect the in vitro activity of recombinant nidovirus RdRps

To establish whether inhibition of RTC activity might be due to a direct effect of Zn^{2+} on nidovirus RdRp activity, we tested the effect of Zn^{2+} on the activity of the purified recombinant RdRps of SARS-CoV (nsp12) and EAV (nsp9) using previously developed RdRp assays [27,28]. Using an 18-mer polyU template, the EAV RdRp incorporated [α-^{32}P]AMP into RNA products of up to 18 nt in length (Fig. 4A). Initiation was *de novo*, which is in line with previous observations and the presence of a conserved priming loop in the nsp9 sequence [28]. Unlike the EAV RdRp nsp9, the *in vitro* activity of the SARS-CoV RdRp nsp12 - which lacks a priming loop - was shown to be strictly primer-dependent [27]. Thus, to study the RdRp activity of SARS-CoV nsp12, a primed polyU template was used (Fig. 4B), thereby allowing us to sample [α-^{32}P]AMP incorporation as described previously [27]. As specificity controls, we used the previously described SARS-CoV nsp12 mutant D618A [27], which contains an aspartate to alanine substitution in motif A of the RdRp active site, and EAV nsp9-D445A, in which we engineered an aspartate to alanine substitution at the corresponding site of EAV nsp9 [28,31]. Both mutant RdRps showed greatly reduced [α-^{32}P]AMP incorporation in our assays (Fig. 4), confirming once again that the radiolabeled RNA products derived from nidovirus RdRp activity.

Addition of $ZnOAc_2$ to RdRp assays resulted in a strong, dose-dependent inhibition of enzymatic activity for both the EAV and

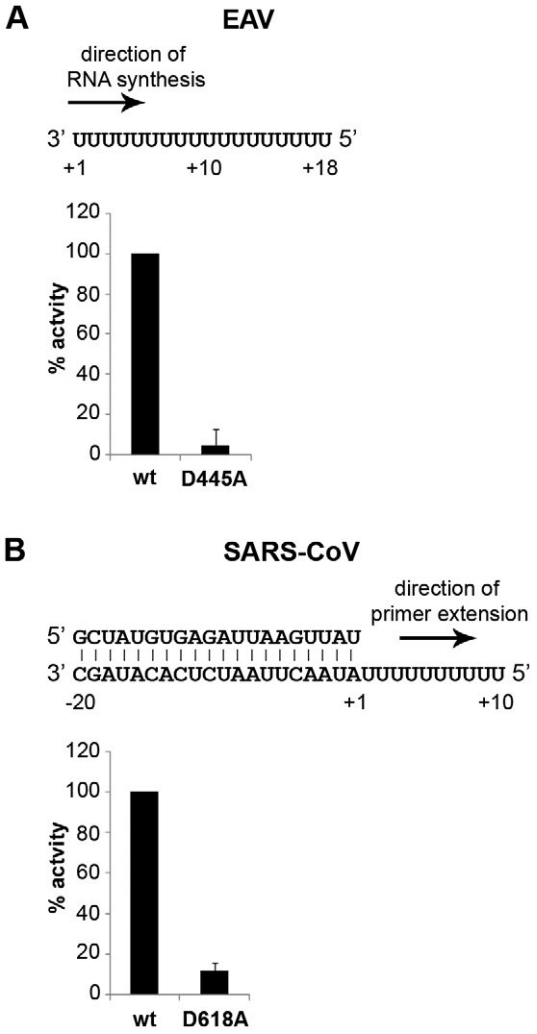


Figure 4. EAV and SARS-CoV RdRp assays with wild-type enzyme and active-site mutants. (**A**) The EAV polymerase was incapable of primer extension and required a free 3′ end and poly(U) residues to initiate. Nucleotide incorporating activity of the wild-type enzyme and D445A mutant of nsp9 on an 18-mer poly(U) template confirmed the specificity of our assay. (**B**) SARS-CoV nsp12 RdRp assays were performed with an RNA duplex with a 5′ U_{10} overhang as template. The bar graph shows the nucleotide incorporating activities of wild-type and D618A nsp12. Error bars represent standard error of the mean (n = 3).
doi:10.1371/journal.ppat.1001176.g004

SARS-CoV enzyme (Fig. 5A and B, respectively), similar to what was observed in RTC assays. In fact, compared to other divalent metal ions such Co^{2+} and Ca^{2+}, which typically bind to amino acid side chains containing oxygen atoms rather than sulfur groups, Zn^{2+} was the most efficient inhibitor of SARS-CoV nsp12 RdRp activity (Supplemental Fig. S1). To test whether, as in the RTC assay, the RdRp inhibition by zinc ions was reversible, RdRp assays were pre-incubated with 6 mM Zn^{2+}, a concentration that consistently gave >95% inhibition. After 30 min, 8 mM MgEDTA was added to both a control reaction and the reaction inhibited with $ZnOAc_2$, and samples were incubated for another 30 min (Fig. 5C). As shown in Fig. 5D, the inhibition of EAV RdRp activity by Zn^{2+} could be reversed by chelation of Zn^{2+} (Fig. 5D; compare lanes 3 and 4). The amount of product synthesized was consistently 60±5% of that synthesized in a 60-min control reaction (Fig. 5D; compare lanes 1 and 4), which was within the expected range given the shorter

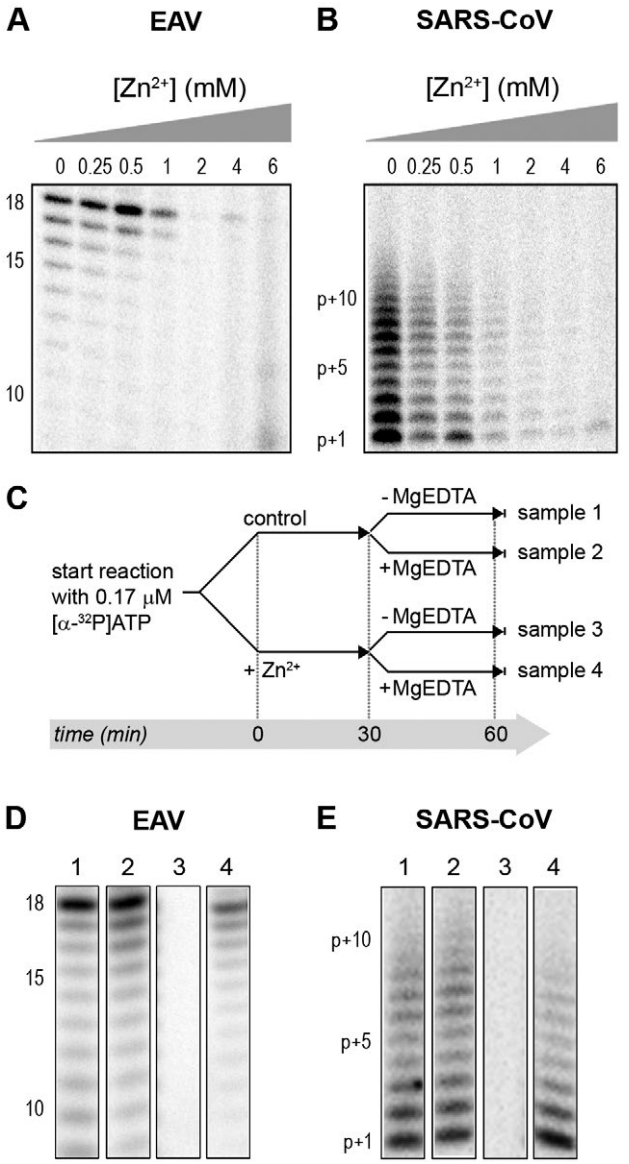


Figure 5. The activity of the RdRps of EAV and SARS-CoV is reversibly inhibited by Zn^{2+}. RdRp activity of purified EAV nsp9 (**A**) and SARS-CoV nsp12 (**B**) in the presence of various Zn^{2+} concentrations, as indicated above the lanes. (**C**) Schematic representation of the experiment to test whether Zn^{2+}-mediated inhibition of RdRp activity could be reversed with MgEDTA. RdRp reactions, either untreated controls (sample 1 and 2) or reactions containing 6 mM Zn^{2+} (samples 3 and 4) were incubated for 30 min. Both Zn^{2+}-containing and control samples were split into two aliquots and 6 mM MgEDTA was added to sample 2 and 4. All reactions were incubated for an additional 30 min and then terminated. Reaction products of the RdRp assays with EAV nsp9 and SARS-CoV nsp12 are shown in (**D**) and (**E**), respectively. Numbers above the lanes refer to the sample numbers described under (C).
doi:10.1371/journal.ppat.1001176.g005

reaction time. The inhibition of the SARS-CoV RdRp was reversible as well. During the 30-min incubation after the addition of MgEDTA, SARS-CoV nsp12 incorporated 40±5% of the label incorporated during a standard 60-min reaction (Fig. 5E). This was slightly lower than the expected yield and may be caused by the elevated Mg^{2+} concentration, which was shown to be suboptimal for nsp12 activity [27] and results from the release of Mg^{2+} from MgEDTA upon chelation of Zn^{2+}.

Differential effect of Zn^{2+} on the initiation and elongation phase of nidovirus RNA synthesis

For EAV, close inspection of the RdRp assays revealed a less pronounced effect of Zn^{2+} on the generation of full-length 18-nt products than on the synthesis of smaller reaction intermediates (Fig. 5A). This suggested that Zn^{2+} specifically inhibited the initiation step of EAV RNA synthesis. To test this hypothesis, an RTC assay was incubated for 30 min with unlabeled CTP (initiation), after which the reaction was split in two. Then, [α-^{32}P]CTP was added to both tubes (pulse), 500 μM Zn^{2+} was added to one of the tubes, and samples were taken at different time points during the reaction (Fig. 6A). Fig. 6B shows that in the presence of Zn^{2+} [α-^{32}P]CMP was predominantly incorporated into nascent RNA molecules that were already past the initiation phase at the moment that Zn^{2+} was added to the reaction. No new initiation occurred, as was indicated by the smear of short radiolabeled products that progressively shifted up towards the position of full-length genomic RNA. This suggested that Zn^{2+} does not affect the elongation phase of EAV RNA synthesis and that it specifically inhibits initiation. This also explains the relatively weak signal intensity of the smaller sg mRNA bands (*e.g.*, compare the relative change in signal of RNA2 to RNA7) produced in the presence of Zn^{2+}, since multiple initiation events are required on these short molecules to obtain signal intensities similar to those resulting from a single initiation event on the long genomic RNA, *e.g.*, 16 times more in the case of RNA7. In contrast to EAV, the effect of Zn^{2+} on RNA synthesis by SARS-CoV RTCs was not limited to initiation, but appeared to impair the elongation phase as well, given that the addition of Zn^{2+} completely blocked further incorporation of [α-^{32}P]CMP when added 40 min after the start of the reaction (Fig. 6C).

In the RdRp assays, the short templates used made it technically impossible to do experiments similar to those performed with complete RTCs. However, we previously noticed that at low concentrations of [α-^{32}P]ATP (~0.17 μM) SARS-CoV nsp12 RdRp activity was restricted to the addition of only a single nucleotide to the primer [27]. EAV nsp9 mainly produced very short (2–3 nt long) abortive RNA products and only a fraction of full length products, as is common for *de novo* initiating RdRps [28]. This allowed us to separately study the effect of Zn^{2+} on initiation and elongation by performing an experiment in which a pulse with a low concentration of [α-^{32}P]ATP was followed by a chase in the presence of 50 μM of unlabeled ATP, which increased processivity and allowed us to study elongation (Fig. 7A and C) as described previously [27]. The results of these experiments were in agreement with those obtained with isolated RTCs. For EAV, with initiation and dinucleotide synthesis completely inhibited by the presence of 6 mM Zn^{2+} (Supplemental Fig. S2A), the amount of reaction intermediates shorter than 18 nt diminished with time, while products from templates on which the RdRp had already initiated were elongated to full-length 18-nt molecules (Fig. 7B, right panel). This was consistent with the observation that the EAV RdRp remained capable of extending the synthetic dinucleotide ApA to trinucleotides in the presence of Zn^{2+} (Supplemental Fig. S2B). Likely due to the absence of reinitiation in the reactions shown in Fig. 7B, the low processivity of the EAV RdRp, and the substrate competition between the remaining [α-^{32}P]ATP and the >200 fold excess of unlabeled ATP, the differences between the 5- and 30-min time points were small. In the absence of Zn^{2+}, the RdRp continued to initiate as indicated by the ladder of smaller-sized RNA molecules below the full-length product (Fig. 7B, left panel) and the time course shown in Supplemental Fig. S2A. In contrast, the addition of Zn^{2+} to a SARS-CoV RdRp reaction also blocked elongation, since

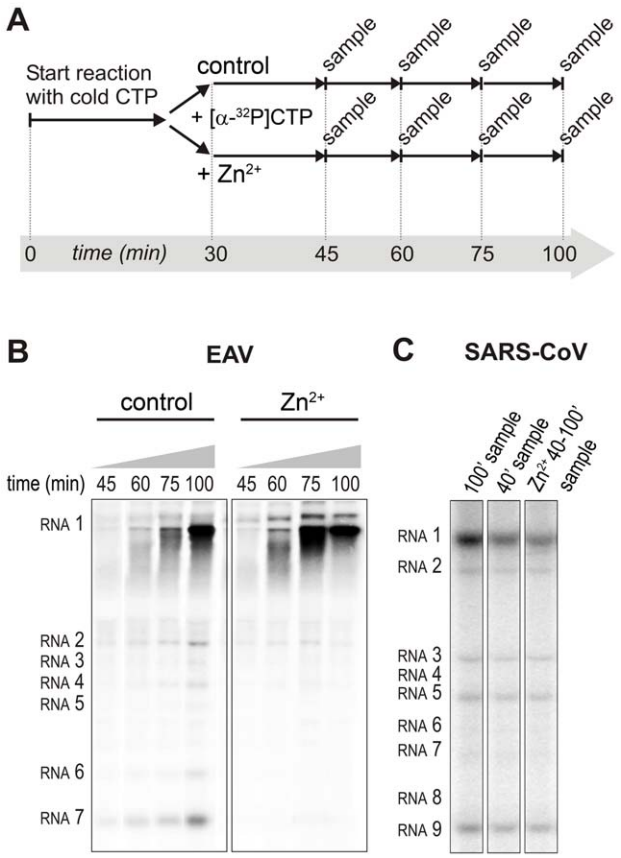


Figure 6. Effect of Zn^{2+} on initiation and elongation in *in vitro* assays with isolated EAV and SARS-CoV RTCs. (**A**) An *in vitro* RTC assay with isolated EAV RTCs was allowed to initiate with unlabeled NTPs (initiation). After 30 min, [α-^{32}P]CTP was added (pulse), the reaction was split into two equal volumes, and Zn^{2+} was added to a final concentration of 0.5 mM to one of the tubes. At the time points indicated, samples were taken and incorporation of [α-^{32}P]CMP into viral RNA was analyzed. (**B**) Radiolabeled EAV RNA synthesized at the time points indicated above the lanes in the presence and absence of Zn^{2+}. (**C**) Radiolabeled RNA synthesized by isolated SARS-CoV RTCs in reactions terminated after 100 (lane 1) and 40 (lane 2) min. Reaction products of a reaction to which 500 μM Zn^{2+} was added after 40 min, and that was terminated at t = 100 are shown in lane 3.
doi:10.1371/journal.ppat.1001176.g006

extension of the radiolabeled primer as observed in the absence of Zn^{2+} (Fig. 7D, left panel) no longer occurred (Fig. 7D, right panel).

Zinc affects SARS-CoV RdRp template binding

To assess whether Zn^{2+} affects the interaction of recombinant SARS-CoV nsp12 with the template used in our assays, we performed electromobility shift assays (EMSA) in the presence and absence of Zn^{2+} (Fig. 8A). To measure the binding affinity of the RdRp for the template, we determined the fraction of bound template at various protein concentrations and observed a 3–4 fold reduction in RNA binding when Zn^{2+} was present in the assay (Fig. 8B). We also assessed whether pre-incubation of the RdRp or RNA with Zn^{2+} was a requirement for this drop in binding affinity, but found no significant difference with experiments not involving such a preincubation (data not shown).

No binding was observed when a similar RNA binding assay was performed with purified EAV RdRp. Likewise, nsp9 did not bind RNA in pull-down experiments with Talon-beads, His_6-tagged nsp9, and radiolabeled EAV genomic RNA or various short RNA templates including polyU, whereas we were able to detect binding of a control protein (SARS-CoV nsp8, which has demonstrated RNA and DNA binding activity [32]) using this assay. It presently remains unclear why we are not able to detect the binding of recombinant EAV nsp9 to an RNA template.

Discussion

Although a variety of compounds have been studied, registered antivirals are currently still lacking for the effective treatment of SARS and other nidovirus-related diseases [33]. RdRps are suitable targets for antiviral drug development as their activity is strictly virus-specific and may be blocked without severely affecting key cellular functions. Several inhibitors developed against the polymerases of e.g. human immunodeficiency virus (HIV) and hepatitis C virus are currently being used in antiviral therapy or clinical trials [34,35,36]. Therefore, advancing our molecular knowledge of nidovirus RdRps and the larger enzyme complexes that they are part of, and utilizing the potential of recently developed *in vitro* RdRp assays [25,26,27,28] could ultimately aid in the development of effective antiviral strategies.

Zinc ions and zinc-ionophores, such as PT and PDTC, have previously been described as potent inhibitors of various RNA viruses. We therefore investigated whether PT-stimulated import of zinc ions into cells also inhibited the replication of nidoviruses in cell culture. Using GFP-expressing EAV and SARS-CoV [29,30], we found that the combination of 2 μM PT and 2 μM Zn^{2+} efficiently inhibited their replication, while not causing detectable cytoxicity (Fig. 1). Inhibition of replication by PT and Zn^{2+} at similar concentrations (2–10 μM) was previously observed for several picornaviruses such as rhinoviruses, foot-and-mouth disease virus, coxsackievirus, and mengovirus [6,7,8,9,10,11].

The inhibitory effect of Zn^{2+} on the replication of picornaviruses appeared to be due to interference with viral polyprotein processing. In infections with the coronavirus mouse hepatitis virus (MHV), Zn^{2+} also interfered with some of the replicase polyproteins cleavages [24], albeit at a much higher concentration (100 μM Zn^{2+}) than used in our studies. Since impaired replicase processing will indirectly affect viral RNA synthesis in the infected cell, we used two recently developed *in vitro* assays to investigate whether Zn^{2+} also affects nidovirus RNA synthesis directly. Our *in vitro* studies revealed a strong inhibitory effect of zinc ions on the RNA-synthesizing activity of isolated EAV and SARS-CoV RTCs. Assays with recombinant enzymes subsequently demonstrated that this was likely due to direct inhibition of RdRp function. The inhibitory effect could be reversed by chelating the zinc ions, which provides an interesting experimental (on/off) approach to study nidovirus RNA synthesis. Addition of Zn^{2+} following initiation of EAV RNA synthesis had little or no effect on NTP incorporation in molecules whose synthesis had already been initiated in the absence of Zn^{2+} (Fig. 6 and 7), indicating that Zn^{2+} does not affect elongation and does not increase the termination frequency, as was previously found for Mn^{2+} [25]. Therefore, Zn^{2+} appears to be a specific inhibitor of the initiation phase of EAV RNA synthesis. In contrast, Zn^{2+} inhibited SARS-CoV RdRp activity also during the elongation phase of RNA synthesis, probably by directly affecting template binding (Fig. 8). In coronaviruses, zinc ions thus appear to inhibit both the proper proteolytic processing of replicase polyproteins [23,24] and RdRp activity (this study). Contrary to the RTC assays, millimolar instead of micromolar concentrations of $ZnOAc_2$ were required for a nearly complete inhibition of nucleotide incorporation in RdRp assays.

It has been well established that DNA and RNA polymerases use a triad of conserved aspartate residues in motifs A and C to bind

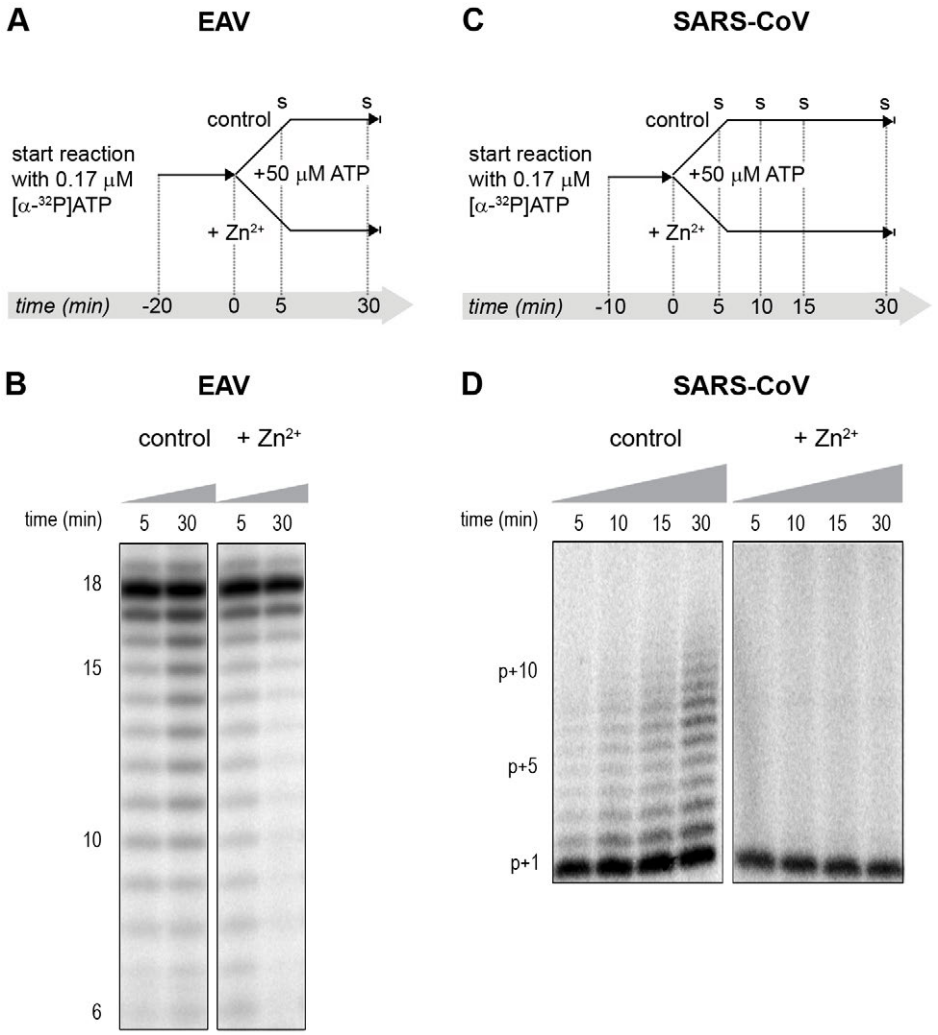


Figure 7. The effect of Zn^{2+} on initiation and elongation activity of purified EAV and SARS-CoV RdRps. (**A**) An EAV RdRp reaction was initiated in the presence of [α-^{32}P]ATP under conditions that do not allow elongation, *i.e.*, low ATP concentration. After 20 min, the reaction was split into two equal volumes, and Zn^{2+} was added to one of the tubes. A chase with 50 μM unlabeled ATP, which allows elongation, was performed on both reactions and samples were taken after 5 and 30 min. (**B**) EAV RdRp reaction products that accumulated in the presence and absence of Zn^{2+} (indicated above the lanes) after a 5- and 30-min chase with unlabeled ATP. The length of the reaction products in nt is indicated next to the gel. (**C**) A SARS-CoV RdRp reaction was initiated in the presence of 0.17 μM [α-^{32}P]ATP, which limits elongation. After 10 min, the reaction was split into two equal volumes, and Zn^{2+} was added to one of the tubes. A chase with 50 μM unlabeled ATP was performed on both reactions and samples were taken after 5, 10, 15, and 30 min. (**D**) SARS-CoV RdRp reaction products formed at the chase times indicated above the lanes in the presence and absence of Zn^{2+}. The length of the reaction products in nt is indicated next to the gel (p is the primer length).
doi:10.1371/journal.ppat.1001176.g007

divalent metal ions like Mg^{2+}, which subsequently coordinate incoming nucleotides during the polymerization reaction [37,38]. Mg^{2+} is also the divalent metal ion that is required for the *in vitro* activity of isolated SARS-CoV and EAV RTCs and recombinant RdRps [25,26,27,28], although *de novo* initiation of EAV nsp9 is primarily Mn^{2+}-dependent. Zn^{2+} could not substitute for Mg^{2+} or Mn^{2+} as cofactor as it was incapable of supporting the polymerase activity of nidovirus RTCs and RdRps in the absence of Mg^{2+} (data not shown), as was also reported for the poliovirus RdRp [39]. Moreover, inhibition of nidovirus RdRp activity by Zn^{2+} was even observed at low concentrations and in the presence of a more than 25-fold excess of Mg^{2+}, suggesting that either the affinity of the active site for Zn^{2+} is much higher or that Zn^{2+} does not compete for Mg^{2+}-binding and binds to another zinc(-specific) binding site in the protein.

Specific protein domains or pockets that contain zinc ions may be involved in protein-protein interactions, protein-RNA/DNA interactions, or conformational changes in enzyme structures. Zinc-binding domains commonly consist of at least three conserved cysteine and/or histidine residues within a stretch of ~10–30 amino acids, such as in zinc-finger motifs and metalloproteases [2,40,41]. However, in RdRps there are only few precedents for the presence of zinc-binding pockets, such as those identified in the crystal structure of the Dengue RdRp [42]. Sequence analysis of the EAV nsp9 amino acid sequence revealed that it lacks patches rich in conserved cysteines and/or histidines. In contrast, inspection of the SARS-CoV nsp12 amino acid sequence revealed two such patches, namely H295-C301-C306-H309-C310 and C799-H810-C813-H816. A crystal structure for nsp12 is presently unavailable, but a predicted structure that represents the C-terminal two-thirds of the enzyme has been published [31]. Interestingly, in this model, C799, H810, C813 and H816 are in a spatial arrangement resembling that of the Zn^{2+}

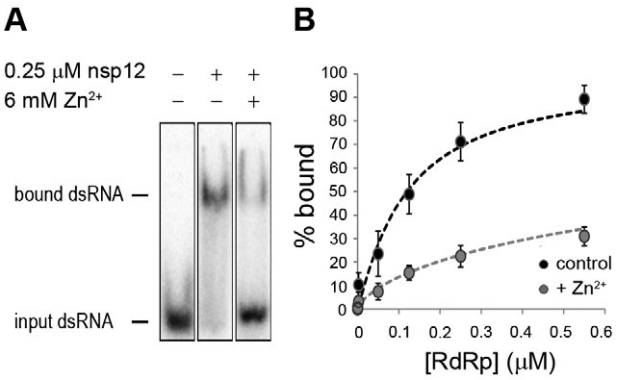


Figure 8. The effect of Zn^{2+} on SARS-CoV nsp12 template binding. (**A**) Electrophoretic mobility shift assay with radiolabeled dsRNA and nsp12 in the presence and absence of Zn^{2+} (indicated above the lanes). The position of unbound and nsp12-bound RNA in the gel is marked on the left of the panel. (**B**) Determination of the nsp12 affinity for RNA in the presence and absence of Zn^{2+}. A fixed amount of RNA was incubated with an increasing amount of nsp12. This revealed a 3–4 fold reduction in the percentage of bound RNA in the presence of zinc ions (grey) relative to the percentage of bound RNA in the absence of zinc ions (black). Error bars represent standard error of the mean (n = 3).
doi:10.1371/journal.ppat.1001176.g008

coordinating residues in the Zn2 zinc-binding pocket found in motif E of the Dengue virus RdRp (see Supplemental Fig. S3). Clearly, an in-depth analysis of nidovirus RdRps, e.g. through structural analysis and subsequent mutational studies targeting aforementioned cysteines and histidines, is required to provide further insight into and a structural basis for the Zn^{2+}-induced inhibitory effects on RdRp activity documented in this study. Such studies may, however, be complicated when Zn^{2+} binding proves to be very transient in nature and not detectable with currently available methods.

In summary, the combination of zinc ions and the zinc-ionophore PT efficiently inhibits nidovirus replication in cell culture. This provides an interesting basis for further studies into the use of zinc-ionophores as antiviral compounds, although systemic effects have to be considered [43,44] and a water-soluble zinc-ionophore may be better suited, given the apparent lack of systemic toxicity of such a compound at concentrations that were effective against tumors in a mouse xenograft model [45]. *In vitro*, the reversible inhibition of the RdRp by Zn^{2+} has also provided us with a convenient research tool to gain more insight into the molecular details of (nido)viral RNA synthesis, and revealed novel mechanistic differences between the RdRps of SARS-CoV and EAV.

Materials and Methods

Cells and viruses

Vero-E6 cells were cultured and infected with SARS-CoV (strain Frankfurt-1; accession nr. AY291315) or SARS-CoV-GFP as described previously [46]. All procedures involving live SARS-CoV were performed in the biosafety level 3 facility at Leiden University Medical Center. BHK-21 or Vero-E6 cells were cultured and infected with EAV (Bucyrus strain; accession nr. NC_002532) or EAV-GFP [29] as described elsewhere [25].

Effect of zinc ions on nidovirus replication in cell culture

One day prior to infection, Vero-E6 cells were seeded in transparent or black (low fluorescence) 96-well clusters at 10,000 cells per well. The next day, cells were infected with SARS-CoV-GFP or EAV-GFP with an m.o.i. of 4, and 1 h p.i. the inoculum was removed and 100 μl of medium containing 2% fetal calf serum (FCS) was added to each well. In some experiments 0–32 μM of pyrithione (Sigma) was added in addition to 0–2 μM $ZnOAc_2$. Infected cells were fixed at 17 h p.i. by aspirating the medium and adding 3% paraformaldehyde in PBS. After washing with PBS, GFP expression was quantified by measuring fluorescence with a LB940 Mithras plate reader (Berthold) at 485 nm. To determine toxicity of $ZnOAc_2$ and PT, cells were exposed to 0–32 μM PT and 0–8 μM $ZnOAc_2$. After 18 h incubation, cell viability was determined with the Cell Titer 96 AQ MTS assay (Promega). EC_{50} and CC_{50} values were calculated with Graphpad Prism 5 using the nonlinear regression model.

RNA templates and oligonucleotides

RNA oligonucleotides SAV557R (5′-GCUAUGUGAGAU-UAAGUUAU-3′), SAV481R (5′-UUUUUUUUUUAUAACUU-AAUCUCACAUAGC-3′) and poly$(U)_{18}$ (5′-UUUUUUUUU-UUUUUUUUU-3′) were purchased from Eurogentec, purified from 7 M Urea/15% PAGE gels and desalted through NAP-10 columns (GE healthcare). To anneal the RNA duplex SAV557R/SAV481R, oligonucleotides were mixed at equimolar ratios in annealing buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl and 5 mM EDTA), denatured by heating to 90°C and allowed to slowly cool to room temperature after which they were purified from 15% non-denaturing PAGE gels.

In vitro viral RNA synthesis assay with isolated RTCs

SARS-CoV and EAV RTCs were isolated from infected cells and assayed for activity *in vitro* as described previously [25,26]. To assess the effect of Zn^{2+}, 1 μl of a $ZnOAc_2$ stock solution was added to standard 28-μl reactions, resulting in final Zn^{2+} concentrations of 10–500 μM. When Zn^{2+} had to be chelated in the course of the reaction, magnesium-saturated EDTA (MgEDTA) was added to a final concentration of 1 mM. After RNA isolation, the ^{32}P-labeled reaction products were separated on denaturing 1% (SARS-CoV) or 1.5% (EAV) agarose formaldehyde gels. The incorporation of [α-^{32}P]CMP into viral RNA was quantified by phosphorimaging of the dried gels using a Typhoon scanner (GE Healthcare) and the ImageQuant TL 7 software (GE Healthcare).

Expression and purification of nidovirus RdRps

SARS-CoV nsp12 and EAV nsp9 were purified essentially as described elsewhere [27,28], but with modifications for nsp9. In short, *E. coli* BL21(DE3) with plasmid pDEST14-nsp9-CH was grown in auto-induction medium ZYM-5052 [47] for 6 hours at 37°C and a further 16 hours at 20°C. After lysis in buffer A (20 mM HEPES pH 7.4, 200 mM NaCl, 20 mM imidazole, and 0.05% Tween-20) the supernatant was applied to a HisTrap column (GE Healthcare). Elution was performed with a gradient of 20–250 mM imidazole in buffer A. The nsp9-containing fraction was further purified by gel filtration in 20 mM HEPES, 300 mM NaCl and 0.1% Tween-20 on a Superdex 200 column (GE Healthcare). The fractions containing nsp9-CH were pooled, dialyzed against 1000 volumes of buffer B (20 mM HEPES, 100 mM NaCl, 1 mM DTT and 50% glycerol) and stored at −20°C. RdRps with a D618A (SARS-CoV) or D445A (EAV) mutation were obtained by site-directed mutagenesis of the wild-type (wt) plasmid pDEST14-nsp9-CH [28] with oligonucleotides 5′-TACTGCCTTGAAACAGCCCTGGAGAGTTGTGAT-3′ and 5′-ATCACAACTCTCCAGGGCTGTTTCAAGGCAGTA-3′, and plasmid pASK3-Ub-nsp12-CHis$_6$ with oligonucleotides 5′-CCTTATGGGTTGGGCTTATCCAAAATGTG-3′ and 5′-CACATTTTGGATAAGCCCAACCCATAAGGA-3′, as described elsewhere [27]. Mutant proteins were purified parallel to the wt enzymes.

RdRp assays with purified enzymes

Standard reaction conditions for the RdRp assay with 0.1 μM of purified SARS-CoV nsp12 are described elsewhere [27]. To study the effect of Zn^{2+} in this assay, 0.5 μl of a dilution series of 0–80 mM $ZnOAc_2$ was added to the 5 μl reaction mixture, yielding final Zn^{2+} concentrations of 0–8 mM. The EAV RdRp assay contained 1 μM nsp9, 1 μM RNA template poly$(U)_{18}$, 0.17 μM [α-^{32}P]ATP (0.5 μCi/μl; Perkin-Elmer), 50 μM ATP, 20 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM KCl, 1 mM $MnCl_2$, 4 mM $MgOAc_2$, 5% glycerol, 0.1% Triton-X100, 1 mM DTT and 0.5 units RNaseOUT. $ZnOAc_2$ was added to the reaction to give a final concentration of 0–6 mM. To chelate Zn^{2+} during reactions, MgEDTA was added to a final concentration of 8 mM. Reactions were terminated after 1 hour and analyzed as described [27].

SARS-CoV nsp12 electrophoretic mobility shift assay

SARS-CoV RdRp was incubated with 0.2 nM 5′ ^{32}P-labeled SAV557R/SAV481R RNA duplex, for 10 minutes at 30°C either in presence or absence of 6 mM $ZnOAc_2$. Reactions were analyzed as described previously [27].

Supporting Information

Figure S1 Effect of various divalent cations on the RdRp activity of SARS-CoV nsp12. Purified recombinant SARS-CoV nsp12 was incubated with a primed template, ATP, and [α-^{32}P]ATP in the presence of either 6 mM Mg^{2+} only (lane 1), and with increasing concentrations of a second divalent metal (M^{2+}), specifically: 2–6 mM Ca^{2+} (lane 2–4), 2–6 mM Co^{2+} (lane 5–7), 2–6 mM Zn^{2+} (lane 8–10), or 2–6 mM Mn^{2+} (lane 11–13). The strongest inhibition was observed for Zn^{2+}. For more details on the SARS-CoV nsp12 RdRp assay, see the main text.
Found at: doi:10.1371/journal.ppat.1001176.s001 (1.55 MB TIF)

Figure S2 Effect of Zn^{2+} on the dinucleotide extension activity of EAV nsp9. Purified recombinant EAV nsp9 was incubated with a U^{18} template in the presence of [α-^{32}P]ATP, ATP, 4 mM Mg^{2+}, 1 mM Mn^{2+}, and 1 μM ApA. (**A**) Reaction mixtures were split into two aliquots, one of which was supplemented with 6 mM Zn^{2+}, and samples were taken at the time points (minutes) indicated above the lanes. In the absence of Zn^{2+}, EAV nsp9 initiates *de novo* and produces di- and trinucleotides, indicated with A2 and A3, respectively. A non-specific band, unrelated to RdRp activity, between A2 and A3 is indicated with an asterisk. In the presence of 6 mM Zn^{2+}, the synthesis of dinucleotides and trinucleotides was blocked. (**B**) When performing the assay described under (A) in the absence of Zn^{2+}, a full-length product of 18 nucleotides is formed. This product is not observed when the assay is performed in the presence of 6 mM Zn^{2+}, but nsp9 was capable of elongating the provided dinucleotide primer ApA into tri- (ApA*pA) and tetranucleotide ((ApA*pA*pA) products (the asterisk indicates radiolabeled phosphates). Due to the absence of a 5′ triphosphate group, these reaction products migrate much slower in the 20% acrylamide and 7 M urea gel used for this analysis. See the main text for additional experimental details on the EAV nsp9 RdRp assay.
Found at: doi:10.1371/journal.ppat.1001176.s002 (2.16 MB TIF)

Figure S3 Putative zinc-binding residues in the predicted structure of SARS-CoV nsp12 and comparison with the structure of the zinc-containing Dengue virus RdRp domain. (**A**) Sequence alignment of coronavirus RdRps showing conservation of four potential zinc-binding residues amino acids (C799-H810-C813-H816 in SARS-CoV; indicated with asterisks) in the C-terminal region of coronavirus nsp12. Black shading indicates complete conservation among coronaviruses. The coronavirus RdRp sequences were aligned with Muscle 3.6. The aligned sequences and NCBI accession numbers are the following: mouse hepatitis virus strain A59 (MHV_A59; NP_068668), human CoV 229E (HCoV_229E; NP_068668), infectious bronchitis virus strain Beaudette (IBV_B; P0C6Y1), bovine coronavirus (BCoV; NP_742138.1), feline coronavirus (FeCoV; YP_239353.1), and SARS-CoV strain Frankfurt-1 (SARS_Fr1; AAP33696). (**B**) Crystal structure of the Dengue virus RdRp domain showing the position of four cysteine and histidine residues that form Zn^{2+}-binding pocket Zn2, located close to motif E (depicted in red). A second Zn^{2+}-binding pocket (Zn1) and the two zinc ions identified in the crystal structure are indicated in blue-gray. (**C**) Predicted three-dimensional structure model of SARS-CoV nsp12 (Xu et al., Nucl. Acids Res. 31: 7117–7130), based on PDB code 1O5S, rendered with Swiss-PdbViewer 4.01 and POV-Ray 3.6. The positions of the conserved cysteine and histidine residues indicated in panel A (C799-H810-C813-H816) close to motif E (depicted in red) and RdRp active-site residues (D618, D760 and D761) are indicated. The spatial arrangement of these cysteines and histidines in this model strikingly resembles the positioning of the metal ion-coordinating residues of Zn-binding pocket Zn2 in the Dengue virus RdRp domain (see panel B).
Found at: doi:10.1371/journal.ppat.1001176.s003 (0.86 MB TIF)

Author Contributions

Conceived and designed the experiments: AJWtV SHEvdW EJS MJvH. Performed the experiments: AJWtV SHEvdW MJvH. Analyzed the data: AJWtV SHEvdW ACS RSB EJS MJvH. Contributed reagents/materials/analysis tools: ACS RSB. Wrote the paper: AJWtV EJS MJvH.

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FOI 26.3133 - Document 5

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Coronavirus biology and replication: implications for SARS-CoV-2

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Abstract | The SARS-CoV-2 pandemic and its unprecedented global societal and economic disruptive impact has marked the third zoonotic introduction of a highly pathogenic coronavirus into the human population. Although the previous coronavirus SARS-CoV and MERS-CoV epidemics raised awareness of the need for clinically available therapeutic or preventive interventions, to date, no treatments with proven efficacy are available. The development of effective intervention strategies relies on the knowledge of molecular and cellular mechanisms of coronavirus infections, which highlights the significance of studying virus–host interactions at the molecular level to identify targets for antiviral intervention and to elucidate critical viral and host determinants that are decisive for the development of severe disease. In this Review, we summarize the first discoveries that shape our current understanding of SARS-CoV-2 infection throughout the intracellular viral life cycle and relate that to our knowledge of coronavirus biology. The elucidation of similarities and differences between SARS-CoV-2 and other coronaviruses will support future preparedness and strategies to combat coronavirus infections.

Coronaviruses (CoVs) are a highly diverse family of enveloped positive-sense single-stranded RNA viruses. They infect humans, other mammals and avian species, including livestock and companion animals, and are therefore not only a challenge for public health but also a veterinary and economic concern. Within the order of *Nidovirales* and the suborder of *Coronavirineae* lies the family *Coronaviridae*. The latter is further specified into the subfamily of *Orthocoronavirinae*, which consists of four genera: *alphacoronavirus*, *betacoronavirus*, *gammacoronavirus* and *deltacoronavirus*. Whereas alphacoronaviruses and betacoronaviruses exclusively infect mammalian species, gammacoronaviruses and deltacoronaviruses have a wider host range that includes avian species. Human and animal coronavirus infections mainly result in respiratory and enteric diseases[1,2].

Human coronaviruses, such as HCoV-229E and HCoV-OC43, have long been known to circulate in the population and they, together with the more recently identified HCoV-NL63 and HCoV-HKU1, cause seasonal and usually mild respiratory tract infections associated with symptoms of the 'common cold'. In strong contrast, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2, which have emerged in the human population over the past 20 years, are highly pathogenic. By infecting bronchial epithelial cells, pneumocytes and upper respiratory tract cells in humans, SARS-CoV, MERS-CoV and SARS-CoV-2 infections can develop into severe, life-threatening respiratory pathologies and lung injuries for which no specific prophylactic or therapeutic treatment has been approved to date.

The initial steps of coronavirus infection involve the specific binding of the coronavirus spike (S) protein to the cellular entry receptors, which have been identified for several coronaviruses and include human aminopeptidase N (APN; HCoV-229E), angiotensin-converting enzyme 2 (ACE2; HCoV-NL63, SARS-CoV and SARS-CoV-2) and dipeptidyl peptidase 4 (DPP4; MERS-CoV). The expression and tissue distribution of entry receptors consequently influence viral tropism and pathogenicity. During the intracellular life cycle (FIG. 1), coronaviruses express and replicate their genomic RNA to produce full-length copies that are incorporated into newly produced viral particles. Coronaviruses possess remarkably large RNA genomes flanked by 5′ and 3′ untranslated regions that contain *cis*-acting secondary RNA structures essential for RNA synthesis. At the 5′ end, the genomic RNA features two large open reading frames (ORFs; ORF1a and ORF1b) that occupy two-thirds of the capped and polyadenylated genome. ORF1a and ORF1b encode 15–16 non-structural proteins (nsp), of which 15 compose the viral replication and transcription complex (RTC) that includes, amongst others, RNA-processing and RNA-modifying

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https://doi.org/10.1038/s41579-020-00468-6

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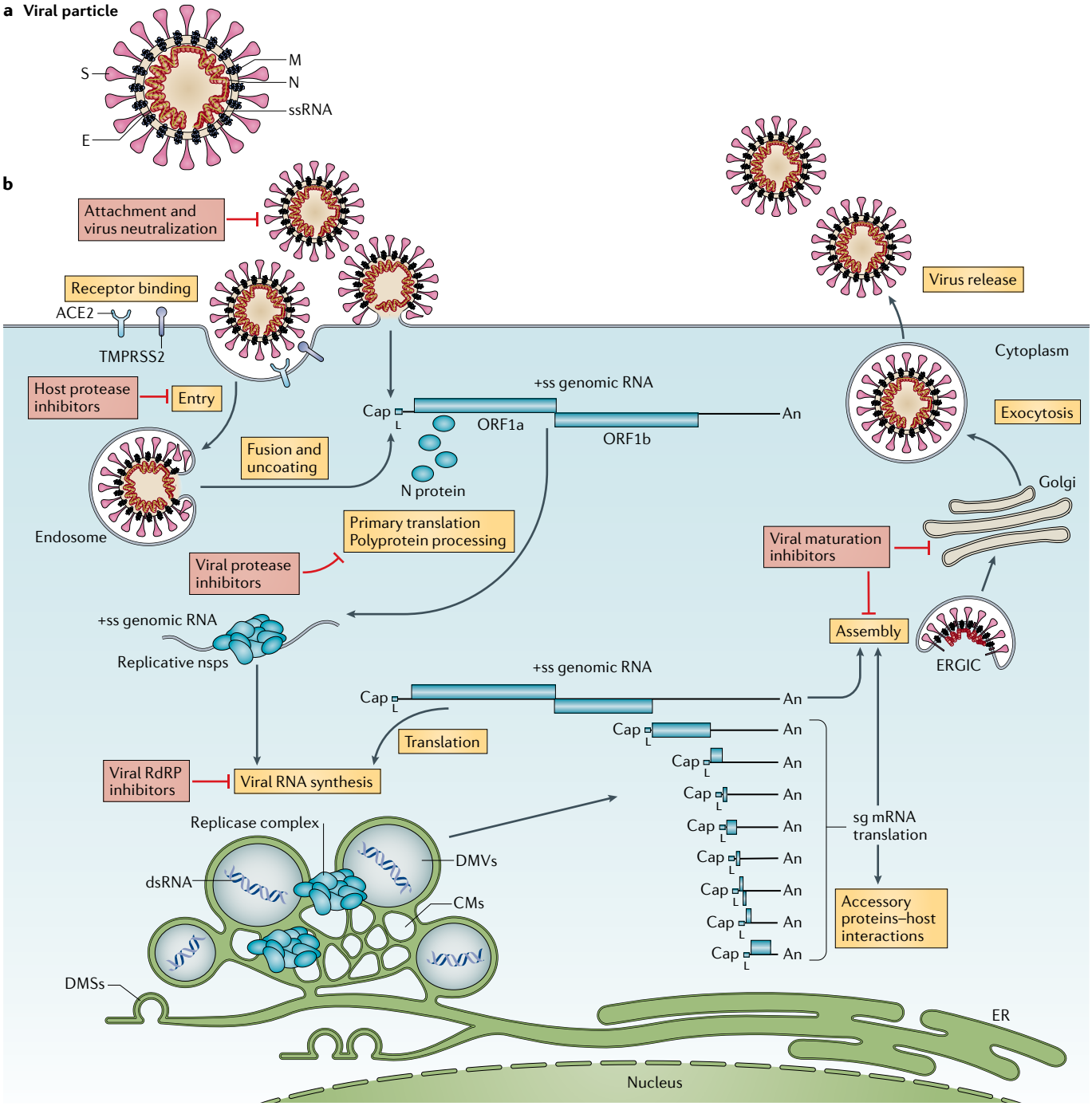


Fig. 1 | **The coronavirus virion and life cycle. a** | The coronavirus virion consists of structural proteins, namely spike (S), envelope (E), membrane (M), nucleocapsid (N) and, for some betacoronaviruses, haemagglutinin-esterase (not shown). The positive-sense, single-stranded RNA genome (+ssRNA) is encapsidated by N, whereas M and E ensure its incorporation in the viral particle during the assembly process. S trimers protrude from the host-derived viral envelope and provide specificity for cellular entry receptors. **b** | Coronavirus particles bind to cellular attachment factors and specific S interactions with the cellular receptors (such as angiotensin-converting enzyme 2 (ACE2)), together with host factors (such as the cell surface serine protease TMPRSS2), promote viral uptake and fusion at the cellular or endosomal membrane. Following entry, the release and uncoating of the incoming genomic RNA subject it to the immediate translation of two large open reading frames, ORF1a and ORF1b. The resulting polyproteins pp1a and pp1ab are co-translationally and post-translationally processed into the individual non-structural proteins (nsps) that form the viral replication and transcription complex. Concordant with the expression of nsps, the biogenesis of viral replication organelles consisting of characteristic perinuclear double-membrane vesicles (DMVs), convoluted membranes (CMs) and small open double-membrane spherules (DMSs) create a protective microenvironment for viral genomic RNA replication and transcription of subgenomic mRNAs (sg mRNAs) comprising the characteristic nested set of coronavirus mRNAs. Translated structural proteins translocate into endoplasmic reticulum (ER) membranes and transit through the ER-to-Golgi intermediate compartment (ERGIC), where interaction with N-encapsidated, newly produced genomic RNA results in budding into the lumen of secretory vesicular compartments. Finally, virions are secreted from the infected cell by exocytosis. Key steps inhibited by compounds that are currently being validated and which represent attractive antiviral targets are highlighted in red. An, 3′ polyA sequence; cap, 5′ cap structure; dsRNA, double-stranded RNA; L, leader sequence; RdRP, RNA-dependent RNA polymerase.

FOI 26-3132 - Document 5

Nested set
Refers to the nested set of coronavirus 5′-coterminal and 3′-coterminal RNAs. Most nidoviruses (*Nidus*, nest, latin) share this mechanism of transcription and are grouped into the order *Nidovirales*.

Zoonotic pathogens
Animal pathogens that can infect and replicate in humans.

enzymes and an RNA proofreading function necessary for maintaining the integrity of the >30 kb coronavirus genome[3]. ORFs that encode structural proteins and interspersed ORFs that encode accessory proteins are transcribed from the 3′ one-third of the genome to form a nested set of subgenomic mRNAs (sg mRNAs). Coronavirus accessory proteins are highly variable sets of virus-specific proteins that display limited conservation even within individual species but they are principally thought to contribute to modulating host responses to infection and are determinants of viral pathogenicity[4,5]. Nevertheless, the molecular functions of many accessory proteins remain largely unknown owing to the lack of homologies to accessory proteins of other coronaviruses or to other known proteins[6].

Despite the previous public health emergencies caused by the SARS-CoV and MERS-CoV outbreaks and the impact of the ongoing SARS-CoV-2 pandemic on society and human health, intervention strategies to combat coronavirus infections are only in their early stages and await proof of clinical efficacy. Their development intimately relies on the deepened understanding of basic mechanisms of coronavirus gene functions as well as of the molecular interactions with host factors. Since the discovery of the first coronavirus (avian infectious bronchitis virus) in the 1930s[7] and the discovery of the first human coronaviruses (HCoV-229E and HCoV-OC43) in the 1960s[8,9], the coronavirus research field has made substantial progress in understanding the basic principles of coronavirus replication and pathogenesis (BOX 1). This advancement was accelerated after the emergence of SARS-CoV in 2002 and MERS-CoV in 2012 and has broadened our view on coronaviruses as zoonotic pathogens that can severely affect human health. Moreover, the unprecedented speed and technical progress of coronavirus research that has become evident in a few months after the appearance of SARS-CoV-2 at the end of 2019 has led to a rapidly growing understanding of this newly emerging pathogen and of its associated disease, COVID-19. In this Review, we discuss key aspects of coronavirus biology and their implications for SARS-CoV-2 infections as well as the treatment and prevention strategies.

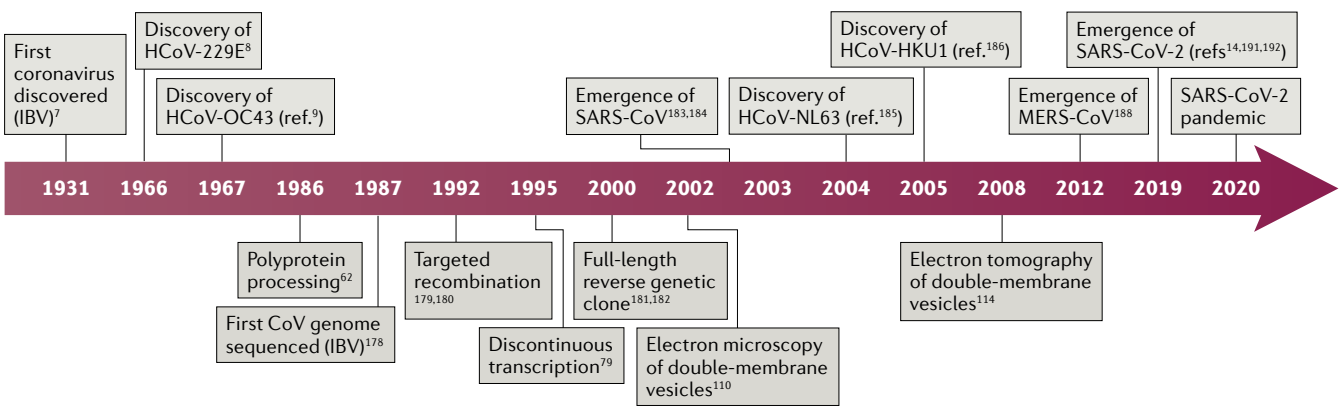
Entry of coronaviruses

Coronavirus S proteins are homotrimeric class I fusion glycoproteins that are divided into two functionally distinct parts (S1 and S2) (FIG. 2). The surface-exposed S1

Box 1 | Milestones in coronavirus discovery and research

Coronaviruses (CoVs) are a large family of viruses long known to infect a wide variety of mammalian and avian species, including livestock and companion animals. In 1931, the avian infectious bronchitis virus (IBV) was the first coronavirus to be discovered[7]. Later, in 1966 and 1967, the first human coronaviruses, HCoV-229E and HCoV-OC43, were discovered[8,9]. The following period was essential in the discovery of research milestones that majorly contributed to coronavirus knowledge: polyprotein processing (1986)[62], first full-length coronavirus genome sequence (1987)[178], first recombinant coronaviruses engineered by targeted recombination (1992)[179,180], discontinuous transcription (1995)[78], full-length reverse genetic clones (2000, 2001)[181,182] and electron microscopy of double-membrane vesicles (2002)[110]. The zoonotic emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) and the subsequent SARS epidemic in 2002–2003 caused 8,000 documented SARS cases, 10% of which had lethal consequences[183,184]. As human-to-human transmission mainly occurred after the onset of symptoms, drastic public health measures, including travel restrictions and isolation of infected patients, succeeded in containing the international spread to limited foyers of infections. The SARS epidemic was followed by an increased amount of virus screening and sequencing, which led to the identification of HCoV-NL63 and HCoV-HKU1 (REFS[185,186]). In contrast to SARS-CoV, HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1 circulate annually and usually cause only mild upper respiratory tract symptoms in immunocompetent individuals[185–187]. In 2008, SARS-CoV-induced double-membrane vesicles were first shown using electron tomography[114]. The emergence of a second highly pathogenic coronavirus of zoonotic origin, MERS-CoV, resulted in more than 2,500 human MERS cases since 2012, associated with virus-induced lung injuries and severe clinical manifestations (36% case fatality rate)[188]. MERS-CoV also originated from bats and established an animal reservoir in dromedary camels[189,190]. Despite sporadic zoonotic transmissions to humans upon prolonged contact and the limited human-to-human transmission, MERS-CoV infections are still detected[190].

Recently, the pathogenic SARS-CoV-2 rapidly spread in the human population after a likely spillover from bats or from a yet unidentified intermediate host[14,191,192]. As of October 2020, more than 40 million COVID-19 cases have been declared in over 200 countries, causing more than 1 million deaths (COVID-19 Dashboard). SARS-CoV-2 targets both upper and lower respiratory tract tissues and efficient human-to-human transmission occurs even before the onset of symptoms[36,193]. Clinical manifestations range from asymptomatic or mild infections to acute lung inflammation and pneumonia, mostly in the elderly and patients with comorbidities[36,194,195].



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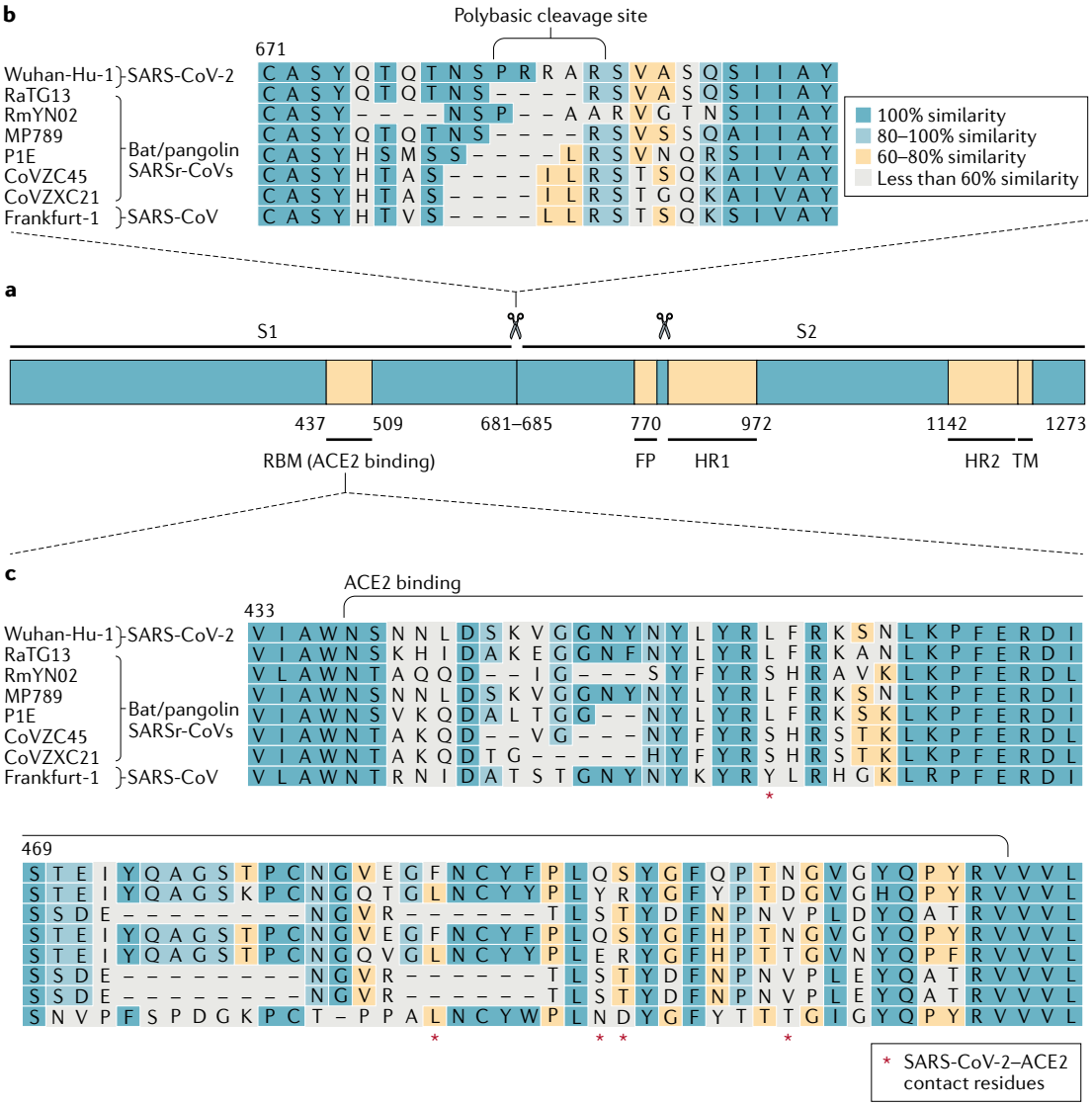


Fig. 2 | **Severe acute respiratory syndrome-related coronavirus spike sequence variation. a** | Schematic illustration of coronavirus spike, indicating domain 1 and domain 2. The receptor-binding motif (RBM) is located on S1 and the fusion peptide (FP), heptad repeat 1 (HR1), HR2 and the transmembrane (TM) domains are located on S2. The cleavage sites are indicated. The colour code designates conserved spike regions surrounding the angiotensin-converting enzyme 2 (ACE2)-binding domain among severe acute respiratory syndrome-related coronaviruses (SARSr-CoVs) and high amino acid sequence variations within the site of receptor interaction. **b** | Amino acid alignment of human SARS-CoV-2 (Wuhan-Hu-1) and SARS-CoV (Frankfurt-1), bat (RaTG13, RmYN02, CoVZC45 and CoVZXC21) and pangolin (MP789, P1E) SARSr-CoVs. The spike gene sequence alignment was performed using MUSCLE and using the default settings and codon alignment, then translated into amino acids using MEGA7, version 7.0.26. The alignment was coloured according to percentage amino acid similarity with a Blosum 62 score matrix. The colour code designates conserved spike regions surrounding the ACE2-binding domain among SARSr-CoVs and high amino acid sequence variations within the site of receptor interaction. The insertion of a polybasic cleavage site (PRRAR, amino acids 681 to 685) in Wuhan-Hu-1 is indicated, and similar insertions are depicted in bat SARSr-CoV RmYN02. **c** | Within the spike sequence, the ACE2 receptor-binding motif (amino acids 437 to 509, black line) is depicted. The spike contact residues for ACE2 interaction are marked with asterisks.

contains the receptor-binding domain (RBD) that specifically engages the host cell receptor, thereby determining virus cell tropism and pathogenicity. The transmembrane S2 domain contains heptad repeat regions and the fusion peptide, which mediate the fusion of viral and cellular membranes upon extensive conformational rearrangements[10–12]. Shortly after the 2002–2003 SARS-CoV outbreak, ACE2 was identified as the functional receptor that enables infection by SARS-CoV[13]. The high genomic and structural homology between the S proteins of SARS-CoV and SARS-CoV-2 (76% amino acid identity) supported the identification of ACE2 as the cell-surface receptor for SARS-CoV-2 (REFS[12,14–16]). Remarkably, essential SARS-CoV contact residues that interact with ACE2 were highly conserved in SARS-CoV-2 as well as in members of the species *Severe acute respiratory syndrome-related coronavirus* that use ACE2 or have similar amino acid side

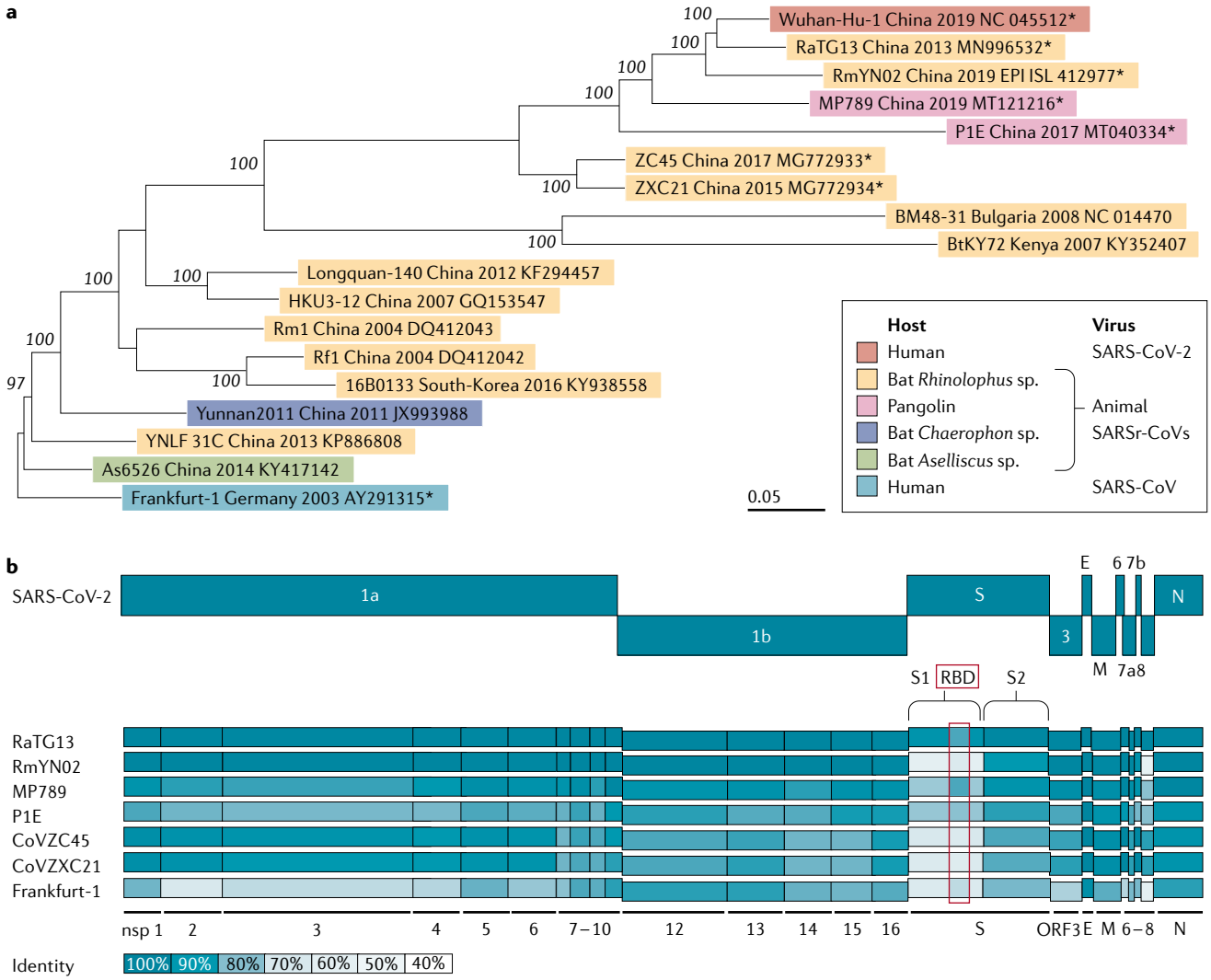
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chain properties[14,15,17–19]. These data were corroborated by the atomic resolution of the interface between the SARS-CoV-2 S protein and ACE2 (REFS[16,19–21]). By contrast, the bat *Severe acute respiratory syndrome-related coronavirus* RaTG13 S sequence (93.1% nucleotide identity to SARS-CoV-2) shows conservation of only one out of six amino acids directly involved in ACE2 binding, even though, based on the entire genomic sequence, RaTG13 is the closest relative of SARS-CoV-2 known to date (96.2%)[14] (BOX 2).

Box 2 | **Diversity of severe acute respiratory syndrome-related coronaviruses**

SARS-CoV-2 belongs to the species *Severe acute respiratory syndrome-related coronavirus* in the subgenus *Sarbecovirus*[1,14,23]. Phylogenetic relationships of representative members of the species *Severe acute respiratory syndrome-related coronavirus* were analysed (sequences retrieved from GenBank and GISAID were analysed using MEGA7 version 7.0.26, asterisks indicate representative viruses further depicted in figure part **b**). Interestingly, SARS-CoV-2 shared 79.6% nucleotide identity with SARS-CoV and close relations to severe acute respiratory syndrome-related coronaviruses (SARSr-CoVs) ZC45 and ZXC21 from *Rhinolophus sinicus*, whereas RaTG13 from *Rhinolophus affinis* showed the highest nucleotide similarity of 96.2%[14,23] (figure part **a**).

Sequence identity differed highly upon comparison of individual genes and domains, indicating frequent recombination events in natural reservoir hosts[14,23,196]. This is exemplified by comparing the nucleotide identity of SARS-CoV-2 with bat coronavirus RaTG13, bat CoV RmYN02, pangolin CoV MP789, pangolin CoV P1E, bat CoV ZC45, bat CoV ZXC21 and human SARS-CoV (Frankfurt-1 strain). Remarkably, in all depicted SARSr-CoVs, the spike gene, a major determinant for zoonotic transmission to humans, showed lower sequence similarity with SARS-CoV-2, thus raising the question of the SARS-CoV-2 origin. Despite the detection of a wide variety of similar bat CoVs in China, SARS-CoV-2 or an immediate precursor have not been found, leaving the role of bats in the emergence of SARS-CoV-2 elusive. Moreover, the environmental separation of bats and humans might favour the existence of an intermediate host, responsible for SARS-CoV-2 adaption and transmission into the human population, just like civet cats were suggested in the SARS-CoV outbreak[197]. The example of pangolin CoV MP789, which shared five essential amino acids for ACE2 binding in the S with SARS-CoV-2 highlights the existence of a variety of unidentified betacoronaviruses in wild-life animals and their roles as possible intermediate hosts[198]. Nevertheless, the number of identified bat SARSr-CoVs represents only a fraction of the existing diversity. The recent identification of bat SARSr-CoVs that can use human ACE2 as an entry receptor (CoV WIV1, CoV bRsSHC014) indicates a possibility of direct cross-species transmission from bats to humans[20,199,200] (figure part **b**).



FOI 26-3132 - Document 5

nutrients

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Review

Zinc as a Gatekeeper of Immune Function

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Received: 27 September 2017; Accepted: 22 November 2017; Published: 25 November 2017

Abstract: After the discovery of zinc deficiency in the 1960s, it soon became clear that zinc is essential for the function of the immune system. Zinc ions are involved in regulating intracellular signaling pathways in innate and adaptive immune cells. Zinc homeostasis is largely controlled via the expression and action of zinc "importers" (ZIP 1–14), zinc "exporters" (ZnT 1–10), and zinc-binding proteins. Anti-inflammatory and anti-oxidant properties of zinc have long been documented, however, underlying mechanisms are still not entirely clear. Here, we report molecular mechanisms underlying the development of a pro-inflammatory phenotype during zinc deficiency. Furthermore, we describe links between altered zinc homeostasis and disease development. Consequently, the benefits of zinc supplementation for a malfunctioning immune system become clear. This article will focus on underlying mechanisms responsible for the regulation of cellular signaling by alterations in zinc homeostasis. Effects of fast zinc flux, intermediate "zinc waves", and late homeostatic zinc signals will be discriminated. Description of zinc homeostasis-related effects on the activation of key signaling molecules, as well as on epigenetic modifications, are included to emphasize the role of zinc as a gatekeeper of immune function.

Keywords: zinc flux; zinc wave; homeostatic zinc signal; immune function; zinc deficiency; signaling pathways

1. Introduction

The transition metal zinc has long been known to be essential for the growth of fungi and development in rodents [1,2]. Symptoms arising from zinc-deficiency in animal studies were reported to be growth failure, loss of hair, testicular atrophy, as well as thickening and hyperkeratinization of the epidermis. Despite these observations, it took a century until zinc was accepted to be essential for human beings [3].

Previously, zinc deficiency was considered to be rare. Nowadays, however, zinc deficiency is known to be very common, especially in developing countries [4]. Worldwide, about two billion people are estimated to be affected by zinc deficiency. In developing countries, zinc deficiency is the 5th leading cause for the loss of healthy life years. In industrial countries, mainly the elderly population is affected by zinc deficiency [5]. Nearly 30% of the elderly population is considered to be zinc deficient. Since zinc homeostasis is known to be important in immunological reactions such as the inflammatory response, and the oxidative stress response, multiple chronic diseases observed in the elderly are probably related to zinc deficiency. Hence, diseases such as Rheumatoid Arthritis, diabetes, atherosclerosis, impaired cognitive function, as well as age-related macular degeneration (AMD) may be due to zinc deficiency, worsening chronic inflammation and triggering oxidative stress [6–9]. Besides the elderly, vegetarians or vegans and patients suffering from renal insufficiency or chronic diarrhea are affected by zinc deficiency [6,9–12]. In humans, clinical manifestations of zinc deficiency are similar to those observed in rodents and include weight loss, growth retardation, atrophy, and immune dysfunction, as well as

FOI 26-3132 - Document 5

increased oxidative stress and a boosted inflammatory immune response [3,13–16]. Dietary insufficiency and/or compromised uptake can result in perturbation of zinc homeostasis in humans [12,17,18].

Besides zinc, vitamins and other transition metals such as copper and iron, as well as calcium are essential for adequate immune function. Deficiency or exceeding levels of those nutrients can be associated with the occurrence of distinct diseases. In this regard, a multitude of diseases such as Rheumatoid Arthritis [19], multiple sclerosis [20–22] impaired cognitive function as seen in Alzheimer's disease [7,23], and AMD [24] were reported to go along with skewed concentrations of the above-mentioned nutrients.

2. Zinc Metabolism and Homeostasis

At 2–3 g in total, zinc is the second most abundant metal in humans and is distributed unequally throughout different organs and tissues. Prostate, pancreas, and bone are considerably high in zinc, containing up to 200 µg/g. In contrast, zinc concentrations in heart, brain and plasma are comparatively low at 1–23 µg/g. Although plasma has only 1 µg/g, it is probably the most important reservoir for zinc homeostasis [5].

Red meat and oysters are rich dietary sources of zinc [5]. Foods prepared from unrefined cereals, legumes, or plant parts rich in phytates decrease zinc bioavailability, by binding zinc very effectively [25]. Zinc is resorbed in the intestine by specific zinc transporting proteins and distributed in the human body. In serum, free zinc is rare since it is largely bound to proteins such as albumin, α_2-macroglobulin (A2M), and transferrin. Albumin binds zinc with a relatively low affinity, A2M with a medium affinity and transferrin with a high affinity. Subcellularly, zinc is distributed between specific zinc-storing vesicles called zincosomes (~50%), the nucleus (~30–40%), and the remainder is distributed between the cytoplasm and other organelles [26,27]. In the cytoplasm, zinc is largely bound by zinc-chelating proteins called metallothioneins (MTs) discovered in 1957 [28]. MTs play an important role in zinc homeostasis by complexing about 20% of intracellular zinc. MT-1 for example binds up to seven zinc ions, making it specifically suitable to function as a zinc buffer [29]. During evolution, cells not only evolved metal-binding proteins to maintain low cytosolic metal ion concentrations to protect themselves against high cytotoxic metal concentrations, but also mechanisms of zinc compartmentalization and zinc sequestration. Modulation of available cytosolic free zinc is facilitated by zinc storage in zincosomes or other organelles such as endoplasmic reticulum (ER) or Golgi apparatus respectively [30]. Zinc distribution between the cytosol an organelles is mediated by specific zinc transporting proteins, such as zinc importers or zinc exporters, or via membrane channels [29,31]. The cytosolic free zinc ion concentration is maintained at remarkably low picomolar to nanomolar concentrations [32]. However, through zinc release from protein and organelles zinc concentrations can increase transiently and locally, enabling zinc to affect gene expression, enzymatic activity, and cell signaling [31,33].

Zinc homeostasis is particularly controlled by 24 zinc transporting proteins, and four MTs (MT1-4), playing coordinated roles in the distribution, transport, and maintenance of intracellular zinc level. MT-1 and MT-2 are expressed ubiquitously throughout the human body, play an important role in liver and kidney function and are inducible by various stimuli such as metal ions, cytokines, glucocorticoids, and oxidative stress, respectively. In contrast, MT-3 and MT-4 are expressed cell specific. MT-3 is primarily expressed in the central nervous system, whereas MT-4 is primarily found in epithelial tissue. The expression of both MT3 and MT4 is very strictly controlled, highlighting distinct functions of MT isoforms in different organs and tissues [34–37].

Based on their membrane topology, zinc transporters are divided into two major families: (1) 14 zinc transporters of the SLC39s family (Zrt (zinc-regulated transporter)-like, Irt (iron-regulated transporter)-like proteins, ZIP 1–14) that increase cytoplasmic zinc and (2) 10 zinc transporters (ZnT 1–10) of the SLC30s family that lower cytoplasmic zinc by transporting zinc either out of the cell or into intracellular organelles [38]. Since zinc transporters are found in a myriad of different cell types in the human body, both on the outer plasma membrane, and on organelle-membranes, such as

FOI 26-3132 - Document 5

mitochondria, Golgi apparatus, lysosomes, and ER, the necessity of a proper zinc level for a specific cellular function is emphasized [38,39]. Moreover, zinc distribution was more recently mentioned in relation to receptors and ion channels other than ZIPs or ZnT, for example voltage-dependent calcium channels, transient receptor potential channels, nicotinic acetylcholine receptors, and glutamatergic receptors. Additionally, diffusion of zinc bound to amino acids is known. However, these mechanisms need to be investigated more in-depth [39].

Interestingly, zinc-dependence was found in all classes of enzymes, i.e., transferases, hydrolases, lyases, isomerases, oxidoreductases, and ligases [40,41]. The physiological importance of zinc was moreover supported by in silico studies which showed that about 10% of the overall human proteome can potentially bind zinc. Zinc binding can be facilitated by a variety of binding motifs including: (1) zinc-finger motifs; (2) Really Interesting New Gene (RING) finger domains; (3) LIM domains composed of two adjacent zinc finger domains; and (4) plant homeodomain (PHD) domains similar to RING fingers [42]. Thus, interaction of the transition metal zinc with proteins is highly complex.

The zinc proteome consists of two major groups, comprising enzymes and transcription factors respectively. Those cover 90% of the overall zinc proteome, indicating that zinc is involved in regulating catalysis and transcription [43,44]. Because zinc is required for catalysis by some enzymes, as well as for maintaining enzyme structure [26], intracellular signaling pathways can be altered due to altered zinc availability, affecting cellular maturation, differentiation, and function [45].

3. Immune Function during Zinc Deficiency

The importance of zinc for proper immune function is best observed in zinc-deficient individuals. Zinc deficiency has been known for 50 years [15] and is associated with skin abnormalities, hypogonadism, cognitive impairment, growth retardation, and imbalanced immune reactions which favor allergies and autoimmune diseases [3,5]. In the case of inherited malfunction of zinc homeostasis, as seen in Acrodermatitis Enteropathica, zinc deficiency can be lethal [17].

Zinc deficiency can be classified by severity and is divided into severe or marginal zinc deficiency, respectively. Severe zinc deficiency is often observed because of malfunction of zinc uptake in the intestine. This is reported in patients suffering from chronic diarrhea, patients being treated with penicillamine, patients receiving parenteral nutrition without zinc, or following excessive alcohol consumption [46–49]. Patients present clinical symptoms such as lymphopenia, decreased ratios of T helper (Th) cells to cytotoxic T cells, decreased natural killer (NK) cell activity, and increased monocyte cytotoxicity. The most severe form of zinc deficiency observed in Acrodermatitis Enteropathica. This zinc malabsorption syndrome is inherited as an autosomal recessive condition and is due to a mutation of the intestinal zinc uptake protein ZIP4 [17,50]. Acrodermatitis Enteropathica is characterized mainly by diarrhea, weight loss, recurrent viral and bacterial infections, dermatitis, hair loss, and neuropsychological disturbances [51]. However, all observed symptoms can be corrected by high dose (1 mg/kg) zinc supplementation [52]. Other zinc transporter-related diseases are discussed later in detail.

Marginal zinc deficiency is characterized by slight weight loss, rough skin, oligospermia, and hyperammonemia [47]. It is probably caused by nutritional zinc deficiency, often seen in vegetarians or vegans, due to the consumption of high levels of zinc-chelating agents in food originating from cereals, legumes, or plant parts. In these foods, lignin and phytates counteract zinc absorption by binding zinc and reducing its bioavailability [10,53]. Nutritionally related marginal zinc deficiency is prevalent in the elderly population. Hence, a correlation between impaired immune function and zinc status is likely in older people [6,54,55]. Within the seventh decade of life, the human immune system undergoes dramatic age-related changes, termed “immunosenescence”. Associated with this condition is an increased incidence of inflammatory disease, most notably cardiovascular diseases, whereas the immunological response to vaccines is typically impaired [56]. The underlying zinc-modulated molecular mechanisms and signaling pathways are discussed below.

FOI 26-3132 - Document 5

The overall frequency of zinc deficiency worldwide is estimated to be higher than 20% [18]. Interestingly, zinc supplementation is already widely practiced and approved for clinical treatment of multiple diseases. Zinc has proven to be very effective for the treatment of pediatric diarrhea, saving millions of children's lives in developing countries such as India [48]. The Food and Drug Administration (FDA) approved zinc supplementation for the treatment of Wilson's disease, a genetic disorder in which copper builds up in the human body [57]. In the elderly population, age-related macular degeneration (AMD) is of frequent occurrence and AMD progress, which can result in blindness, can be treated successfully by zinc supplementation [58,59]. Furthermore, not only the very young or elderly benefit from zinc supplementation, as shown by studies with patients suffering from: (1) viral infections, e.g., the common cold, diarrhea, chronic hepatitis C, or human immunodeficiency virus (HIV); (2) bacterial infections such as *shigellosis* or *Helicobacter pylori*; (3) parasitic infestations such as acute cutaneous leishmaniosis or malaria; (4) autoimmune diseases such as Type 1 Diabetes Mellitus (T1DM) and Rheumatoid Arthritis; and (5) transplant rejections [60]. This widespread variety of clinical manifestations makes zinc deficiency a serious nutritional problem. However, to date, no reliable biomarker to assess zinc status exists [61]. Thus, zinc deficiency is difficult to diagnose. Overall, zinc contributes to the overall regulation of immune cell function, influencing several signaling pathways. Hereby, zinc acts in a direct manner by binding reversibly to regulatory sites in signaling proteins, or indirectly by influencing enzymes such as phosphatases which are a component of and regulate signaling pathways [45].

Zinc homeostasis is essential for multiple aspects of the immune system including hematopoiesis, cell maturation and cell differentiation, cell cycle progression, and for the proper function of immune-cells [62]. During inflammation, adequate zinc status is essential since in acute phase responses zinc is transiently transferred from serum into organs, especially the liver, causing transient serum hypozincemia. This transient loss of serum zinc is eventually rebalanced during resolution of the inflammatory response. Here, zinc is probably released from tissue into serum. One proposed reason for this complex mechanism is to act as a danger signal for immune cells [63]. Since extracellular microorganisms are also dependent on zinc availability, zinc sequestration by the human immune system helps to combat invading pathogens. This is facilitated due to expression of pro-inflammatory acute phase proteins including interleukin (IL)-6, which upregulates expression of zinc binding peptides such as MTs and A2M [64]. In immune cells, on the one hand, increased intracellular zinc levels can intoxicate engulfed pathogens and act cytoprotectively by neutralizing reactive oxygen species (ROS) and nitrogen species (RNS). In general, zinc homeostasis and zinc signals are crucial to counteract inflammatory diseases, and the correlation of undernourishment with severe inflammatory diseases is accompanied by prolonged and severe forms of serum hypozincemia. In the literature, it has been suggested that hypozincemia goes along with elevated inflammatory mediators, e.g., ROS, and antimicrobial peptides such as calprotectin or matrix metalloproteases (MMP), causing tissue injury, especially in liver, lung, and spleen [63,65,66].

In general, cellular function, such as the intracellular killing of harmful pathogens, cytokine production as well as ROS production, are dependent on zinc and are impaired due to zinc deficiency. Zinc deficiency also adversely affects the maturation and function of T and B cells, which occurs through dysregulation of basic biological functions at the cellular level, described later in this review in more detail. For T cells, a disturbed ratio of Th1 and Th2 cells in favor of Th2-driven allergic reactions is a well-known consequence of zinc deficiency [67]. Zinc flux and homeostatic zinc signals, as defined below, are highly important for adequate T cell differentiation, and this observed malfunction can be reversed by zinc supplementation [68–71]. Moreover, the pro-tolerogenic immunoreaction is triggered by long-lasting changes in intracellular zinc levels due to induction of regulatory T cells (Treg) cells and dampening of pro-inflammatory Th17 and Th9 cells [72–75].

T cell development is strongly dependent on DC activation. Interestingly, zinc signals were recently shown to induce a tolerogenic DC phenotype in vitro and in vivo. Herein, zinc suppressed MHC-II expression and enhanced programmed cell death 1 ligand 1 (PD-L1) and PD-L2 expression

FOI 26-3132 - Document 5

resulting in the manipulation of the Treg/Th17 balance in favor of Treg cell development [76]. Moreover, on the molecular level, zinc inhibits the IL-6-induced STAT3 signaling cascade essential for Th17 development [74]. Another potential target of zinc-mediated Th17 manipulation might be found on the epigenetic level since several epigenetic enzymes as (de-)acetylases, and (de-)methylases are regulated in a zinc dependent manner [72,77].

A malfunctioning adaptive immune system has been observed in the elderly population, whereby secretion of pro-inflammatory IL-6 is pathologically elevated, while T cell activation was reduced, as were responses to stimulation or vaccination [6,56,68,70,78,79]. Interestingly, all these pathologies can be improved due to zinc supplementation, highlighting the significance of zinc for a well-balanced immunoreaction.

Regarding innate immunity, zinc deficiency leads to prioritization of maturation of innate immune cells such as monocytes. In this case, differentiation was promoted by the reduction in the concentration of intracellular free zinc [80]. This was facilitated by induced expression of the zinc-binding heterodimeric protein calprotectin [81]. In general, calprotectin was also highly expressed in neutrophils [81,82]. Recently, zinc deficiency has been shown to negatively influence critical neutrophil functions such as phagocytosis, oxidative burst, degranulation, cytokine production, chemotaxis, and neutrophil extracellular trap (NET) formation [83,84]. These observations are in line with an impaired ROS production during zinc deficiency, which is needed for NET formation, and for intracellular killing of phagocytosed pathogens by neutrophils [85,86].

Furthermore, NK cell function was weakened when zinc signals are absent. In this regard, recognition of major histocompatibility complex class I (MHC-I) on target cells, as well as lytic activity, was decreased [87–90]. In contrast, zinc supplementation increased differentiation of CD341 progenitors toward NK cells and their cytotoxic activity [91], as well as NK killing activity, and intracellular perforin concentrations [90].

Besides cellular immune responses, zinc is also indispensable for proper RNA transcription, DNA synthesis, as well as cell survival [27,92]. In relation to cell survival and apoptosis, adequate intracellular zinc levels are needed because apoptosis is triggered by zinc deficiency. Furthermore, cytokine function and secretion are adversely affected by zinc deficiency impairing the function of the basic messengers of the immune system. Thus, zinc is crucial for the appropriate development and function of the whole immune system including innate as well as adaptive immunity, and the affected signaling cascades and networks are described in detail below.

4. Types of Zinc Signals

As indicated earlier, intracellular as well as extracellular zinc is usually bound to protein. From protein, zinc can readily be made available but also made unavailable. About 10% of total human proteins bind some zinc throughout the lifetime of the protein, as it is essential for catalytic or structural functions. In these cases, zinc is not released under normal circumstances. Other proteins revealed rapid association and dissociation rates for zinc, short enough for zinc to regulate processes such as pathogen sensing, intracellular transportation and signaling. This pool of zinc is called “free”, “labile” or “mobile” zinc, describing loosely bound or unbound ions that are thermodynamically available for cellular metabolism. Coordination chemistry of those “mobile” zinc ions has been barely explored [30]. Thus, it is difficult to create artificial substances that can deliver zinc or chelate it away from protein. This explains why there is so far no clear answer to the question, which zinc compounds are best to use for zinc supplementation. In addition, it underlines why there is still no reliable method to measure zinc status of individuals and the immense discrepancies between studies even if experimental set ups seem to only differ slightly.

Zinc is released from bound protein during regular protein turnover. It is mobilized from protein by chemical processes, and can be mobilized between the extracellular and intracellular, organellar or vesicular compartments [30]. Efficient zinc buffering inside the cells, to avoid zinc-intoxication and zinc-deficiency, is regulated by coordinating expression of zinc binding proteins and zinc

FOI 26-3132 - Document 5

transporters [37]. This includes so-called muffling, where proteins transiently bind zinc and transport it to vesicles [29]. To be correct, zinc ions are never completely "free" inside the cell but always complexed with amino acids, phosphates or other low molecular weight ligands. However, "free zinc" will be used here, to distinguish the pool of zinc that is available for signaling from zinc stably bound to protein and therefore unavailable. This is the term, generally used in literature in this field, although it is not a satisfactory definition in the chemical sense [30].

After stimulation of cells, intracellular zinc fluctuation, or in short "zinc flux", has been observed, suggesting a function for zinc as a second messenger [93–95]. In myeloid cell lines, primary monocytes and granulocytes, a zinc flux can be induced by lipopolysaccharide (LPS) or phorbol-12-myristate-13-acetate (PMA), which was not detected in lymphoid cells [83,94]. Furthermore, Fc-epsilon receptor I (FcεRI) activation caused a zinc flux in mast cells [96]. Increased intracellular zinc in monocytes was also detected after stimulation with monocyte chemoattractant protein (MCP-1), tumor necrosis factor (TNF)α, insulin and Pam3CSK4 [97]. Using various ligands for toll like receptors (TLR) including Pam3CSK4 (TLR1/2), *Listeria monocytogenes* (TLR2), flagellin (TLR5), FSL-1 (TLR6/2), ssRNA40 (TLR7) and inhibitory oligonucleotides (ODN) 1826 (TLR9) all increased intracellular zinc in murine macrophages and primary human monocytes [94,97]. In these cases, zinc was mostly shown to be increased, but a decrease might occur as well. Zinc can transduce the extracellular stimulus into an intracellular signaling event. Release of zinc from the endoplasmic reticulum has been shown to be inducible by some hormones, similarly to what has been described for calcium [98]. Another source of zinc is zinc-binding-proteins as already indicated. Here, MTs play a decisive role, as they bind up to seven zinc ions, which can be released rapidly. Zinc ions can be released from their coordination environment with sulfur donors. Zinc is released from cysteine in proteins, suggesting that a redox signal can be "translated" into a zinc signal [99], which we will return to later in this review.

As the zinc flux occurs within seconds to minutes of stimulation, it is not due to changes in gene expression, but alteration of activity of existing agents. Figure 1 illustrates that not only fast zinc fluxes exist, but also a so called "zinc wave", which occurs within a few minutes. For the zinc wave, the influx of calcium is essential. This has been described after cross-linking of FcεRI in mast cells [96]. Furthermore, a delayed signal occurs a few hours after stimulation. Regarding this zinc signal, a certain stimulus activates expression of genes involved in zinc metabolism, including zinc transporters and zinc binding proteins, causing alteration of intracellular zinc levels some time after the initial stimulus. This third type of zinc signal is said to have mostly homeostatic functions and will therefore be named accordingly here. Here, intracellular zinc levels are changed long-term, i.e., permanently elevated or decreased compared to the original concentration measured inside the cell before stimulation occurred. The homeostatic zinc signal was shown to be important for major cellular changes such as the process of maturation and differentiation of myeloid and dendritic cells [80,100]. In B and T cells, stimulation induces a sustained increase in intracellular zinc due to downregulation of ZnT1, ZnT 4–7 and upregulation of ZIP6, ZIP8, and ZIP10 [60,101]. When ZIP6 and ZIP8 were silenced, cytokine production and proliferation of T cells was blocked [71,101,102]. Similarly, BCR-induced signaling was disrupted in cells from ZIP10 knockout mice [103]. Various activation signals, including mediators of diseases, change the expression of MTs, enabling regulation of zinc homeostasis in the long term as well [80,104].

FOI 26-3132 - Document 5

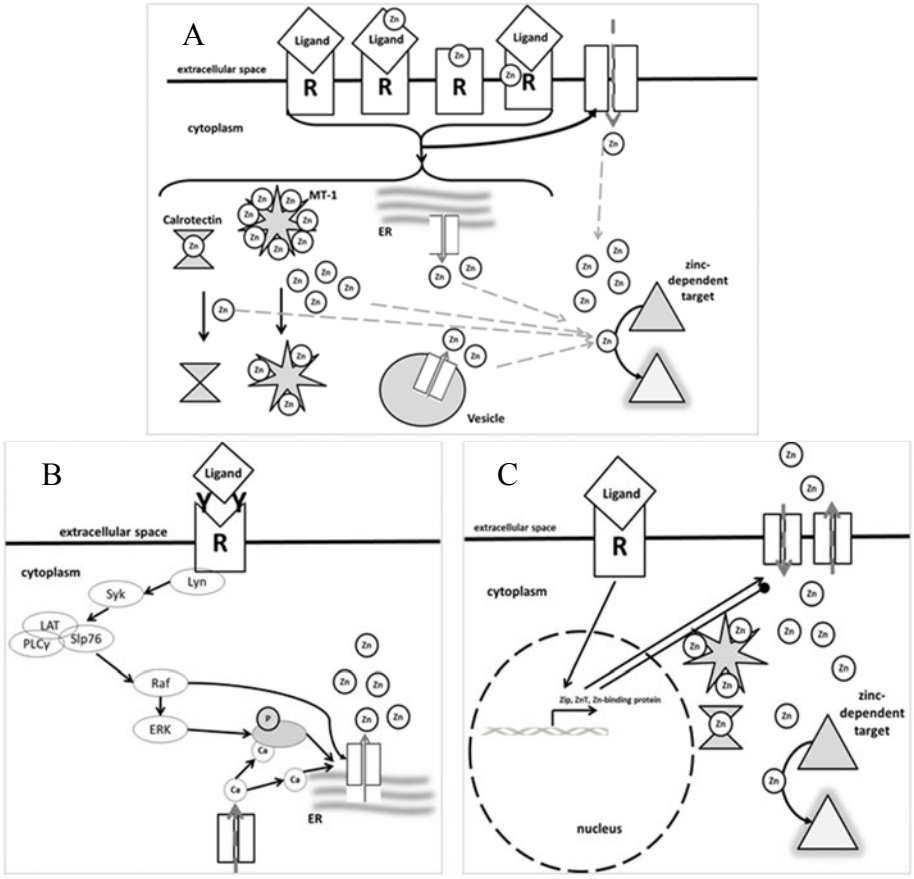


Figure 1. Different Types of Zinc Signals: (**A**) Zinc Flux, as observed after receptor triggering (e.g., binding of lipopolysaccharide (LPS) to Toll like receptor (TLR)4), is generated within seconds. (**B**) A Zinc Wave, as is induced via immunoglobulin receptors and involving calcium flux, can be observed within a few minutes. (**C**) Homeostatic Zinc Signals, for example as observed after LPS stimulation of dendritic cells, take a few hours to be established and involve the expression of zinc transport and binding proteins. For explanations see the text. Abbreviations: ER: endoplasmic reticulum; ERK: extracellular signal-regulated kinase; MT: metallothionein; PLC: phospholipase c; R: receptor; Slp76, SH2 domain-containing leukocyte protein, 76 kD. Modified after [62,96,105].

5. Effects of Zinc in Immune Cell Signaling

Changes in extracellular zinc levels, such as serum hypozincemia during acute phase reactions, have been suggested to activate immune cells, functioning as a "danger signal". In addition, cytokines, integrin binding, growth factors and other immune cell receptor ligands trigger intracellular zinc flux. In recent years more and more regulatory pathways have been demonstrated in various immune cells to directly or indirectly involve zinc signaling. The following section provides a summary of recent developments, concentrating on the major mechanisms in immune cells, and for further information the reader is referred to the extensive literature on this topic [39,62,103,106]. Exemplarily, we will describe briefly key signaling pathways for cells from the adaptive as well as the innate immune system. General concepts, such as the effect of intracellular zinc concentrations on the activities of phosphodiesterases (PDE), phospho tyrosine phosphatases (PTP) and their antagonists the tyrosine kinases (TK), or the translocation of signaling molecules and transcription factors such as NFκB to the nucleus, can probably be extrapolated to other examples of receptor-induced signaling pathways. However, cell type dependent exceptions to the paradigm might exist, underlining the importance of testing the general concepts for each individual cell type and pathway.

FOI 26-3132 - Document 5

The ideal immune response should be fast and specific against each kind of molecular pattern associated with extracellular or intracellular pathogens, and degenerate or damaged cells. Instead of assigning all the necessary functions to one single cell type, the immune response is optimized by the cooperation of various cell types, supported by soluble mediators with distinct functions, roughly separated into innate, fast responding cells and highly specific adaptive immune cells. Quiescent cells circulate in the bloodstream and enter the tissues after activation in case of an infection. Here, firstly innate immune cells, composing mainly granulocytes and monocytes, but also NK cells, mast cells and dendritic cells are recruited. These cells can recognize general pathogen-associated molecular patterns (PAMPs) via surface receptors denoted as pattern recognition receptors (PRRs). Examples of PRRs include the Toll-like receptors (TLRs), with at least 10 members in humans, nucleotide-binding oligomerization domain-like receptors (NLRs), and retinoic acid-inducible gene-I-like receptors (RLRs). Receptor triggering activates signaling pathways and subsequent anti-microbial activities such as degranulation, phagocytosis and pathogen-killing, the presentation of the antigen to other (adaptive immune) cells and cytokine production [107]. Adaptive immune cells, resembling T- and B-lymphocytes, are in contrast highly specific, but require a longer time to become fully active. Each cell has a receptor, called B cell receptor (BCR) or T cell receptor (TCR), and thereby specificity for a certain antigen [108,109]. Triggering of the naïve cell by its matching antigen activates intracellular signaling and causes the proliferation of this particular cell clone and its differentiation into an effector or memory cell. Binding of co-stimulatory molecules, for example interleukins or interferons, to their receptors is necessary for the fine-tuning of the pathogen-specific response. Adaptive immune cells carrying receptors for autoantigens, are selected and silenced.

5.1. Zinc Signals Can Regulate Phosphatase and Kinase Activities

The translation of an initial stimulus, detected by a cell surface receptor, in to gene expression in the nucleus is largely mediated by transferring phosphate residues from one signaling molecule to the next in a consecutive cascade [110]. Here, tyrosine, serine and threonine residues in signaling proteins are the acceptors of the phosphate residues in most cases. Phosphorylation is mediated by protein kinases, while protein phosphatases are responsible for removing phosphates. Combining results for tyrosine phosphorylation of multiple studies, one can assume that phosphorylation is balanced by zinc levels, as zinc affects the decision over the activity of protein tyrosine kinases (PTKs) and phosphatase (PTPs). Phosphorylation of a target protein can result in an activation or an inactivation of that protein and each case must be specifically examined [111].

Several PTKs, belonging largely to the Src family, were shown to act in a zinc–dependent manner. Here, zinc supported the phosphorylation of target molecules. Examples include p60c-Src as found in Alzheimer's patients, anaplastic lymphoma kinase, which is a member of the receptor tyrosine family, Bruton's kinase (BTK) and also mitochondrial Src tyrosine kinases, showing that kinases from various cellular compartments can be affected by zinc levels [112–116].

A group of protein kinases important for signaling in immune cells is the protein kinase C (PKC) family, reported to be zinc metallo-enzymes. These are serine/threonine kinases of several isoforms, including classic PKC, which are activated by cofactors including diacylglycerol (DAG) or calcium, novel PKCs that do not bind calcium but only DAG and atypical PKCs which bind neither of these co-factors. Zinc was reported to be important for PKC's structure. Approximately four zinc ions were found associated with each PKCα subunit and similar values have been reported for PKCβII and PKCγ. This matches well with PKC's C1 domain forming four Cys3His binding motifs, probably occupied by zinc. As this domain is conserved across all PKC isoforms, zinc might be structurally important for all PKCs [117]. Whether more zinc ions can be bound is questionable, but a release of zinc from PKC after incubation with lipid second messengers or due to thiol oxidation has been noted [118]. Activation of PKC, especially by homeostatic zinc signals, has been shown. Thus, amongst others, binding of phorbol esters to the cytoskeleton and the plasma membrane has been found to require zinc [119]. IKK and NFκB activity was decreased in macrophages negative for PKCε. LPS stimulation

FOI 26-3132 - Document 5

of such PKCε knockout macrophages, showed lower ERK and p38 phosphorylation compared to wildtype controls, suggesting that here, the zinc signal alters signal transduction via PKC [120].

In this context, the tight interrelation of zinc homeostasis with redox metabolism should be mentioned. PKC, activated through PMA, subsequently causes the production of reactive oxygen species by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Induction of a zinc flux by PMA was mentioned earlier [93]. When NADPH oxidase was inhibited, no increase in intracellular zinc was observed after PMA stimulation [66]. Incubation of cells with H_2O_2 on the other hand, elicited elevated intracellular zinc concentrations, suggesting an association of ROS production with the occurrence of a zinc signal. In this case, the source of zinc might be PKC itself, or zinc-binding proteins such as MT, both known to release zinc when oxidized. Alternatively, zinc might be released from cellular compartments in an oxidant-sensitive way [71,94,101,121,122]. Maybe as a feedback mechanism, zinc affects ROS production as well. Incubation of cells with a high concentration (100 μM) of the zinc chelator TPEN caused altered mobilization of PKC between plasma membrane and the cytoskeleton and inhibition of PKC activity [93,123,124]. When myeloid cells were depleted of zinc by incubation in zinc-deficient medium, basal and stimulation-induced ROS levels were increased [66]. Increased ROS production in zinc deficient neutrophils was also observed [66,84,125–127]. ROS as well as zinc are essential second messengers for the activation of various functions in neutrophils and other immune cells [83].

Activity of PKA and PKG (both serin/threonine kinases) are mainly affected by levels of the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), respectively. Increased cGMP additionally cross-activates PKA. PDEs are zinc dependent and hydrolysis of both cAMP and cGMP has been shown to be affected by zinc. Zinc is tightly bound to the catalytic center of PDE, but PDE also has an inhibitory site, where available zinc binds if present in elevated concentrations [128,129]. Furthermore, it was shown that zinc affects LPS-induced expression of certain PDEs, including PDE4B [130]. Therefore, cAMP and cGMP levels increased if high zinc concentrations were available because hydrolysis by PDE was inhibited. This was shown for PDE 4, 3, and 1, which were all inhibited by zinc in monocytes. Zinc flux activated adenylate cyclase (AC) while the enzyme was inhibited by homeostatic zinc signals, probably as a feedback mechanism to resolve stimulation-mediated effects. Alterations in guanylate cyclase (GC) activity were only affected by the zinc flux [131,132]. Downstream targets of PKA include Raf-1, responsible for NFκB activation, while PKG targets members of the MAPK family, offering another mechanism of zinc's effect on signaling, which remains to be investigated in more detail [133].

As suggested earlier, zinc seems to balance phosphorylation of signaling molecules in direct and indirect ways, altering the activities of kinases and their antagonists, the phosphatases. PTPs are largely regulated by proteolysis, dimerization, the redox state of the cell and phosphorylation and dephosphorylation. Zinc is one more regulator to add to the list, as was shown amongst others for PTP1B (IC_{50} (zinc) = 3–17 nM), SHP-1 (IC_{50} (zinc) = 93 nM, SHP-2 (IC_{50} (zinc) = 1–2 μM), PTPRB (IC_{50} (zinc) = 98 pM), PTEN (IC_{50} (zinc) = 0.6 nM) [134,135] and the same might be true for several others from the list of 107 PTPs in humans. IC_{50} values all fit well with the physiological concentration of zinc within cells, so that regulation of their activity by zinc signals is plausible. Suggested mechanism underlying the regulation of PTP activity by zinc include the displacement of co-factors by zinc and the direct interaction of zinc with the catalytic center of PTPs [136]. Prolonged protein tyrosine phosphorylation was described in many signaling pathways of immune cells [94,97,137]. An effect of zinc has thus not been investigated in all of them, but its involvement in regulating those signaling pathways is likely. Similar inhibitory effects of zinc were found for MAPK phosphatases (MKP), which are closely related to PTPs, but largely affected the phosphorylation of threonine residues [138] and for calcineurin (CN), a threonine/serine phosphatase (IC_{50} (zinc) = 10 nM–10 μM). Because the range of zinc IC_{50} for this enzyme is large, the effect of an intracellular zinc signal might be questionable. However, in vitro investigations in T cells revealed

FOI 26-3132 - Document 5

that CN's activity was indeed decreased by zinc. In this case, replacement of catalytically active nickel by zinc was found as one underlying mechanism [101,139,140].

Regarding intracellular signaling, the major targets of protein kinases and protein phosphatases are members of the mitogen activated protein kinase (MAPK) family. Here, phosphorylation of extracellular Signal-regulated Kinase (ERK), MAPK/Erk kinase (MEK), p38 has been shown to vary depending on the zinc status of a cell [134,136,141–143]. Long-term zinc deficiency was associated with increased p38 activity in myeloid cells [66]. In contrast, zinc activated ERK in fibroblasts [144]. Differences in effect are most probably due to divergent experimental conditions and cell types, such as duration and intensity of zinc supplementation or deficiency. Another group of signaling molecules whose phosphorylation status and activity is altered by zinc are STAT molecules, as was demonstrated for STAT1, STAT3, and STAT6 [145,146]. Investigations of the role of zinc signals in the regulation of STAT5 activity showed varying results and may depend on the signaling pathway [71,135,147].

5.2. Effects of Zinc Finger Proteins on Signal Transduction

When it comes to homeostatic zinc signals, the zinc-finger protein A20 is of interest, being significantly involved in the resolution of infections. Induction of A20 expression by zinc signals, which resulted in increased intracellular A20 protein levels, was observed in pre-monocytic and endothelial cells as well as in other immune cells. In TLR- and TNFR-triggered signaling pathways, A20 was shown to down-regulate signaling, thereby ameliorating inflammation. TNF receptor associated factor (TRAF) 6 is de-ubiquinated by A20, abrogating TLR-induced signaling. A20 can remove ubiquitin from receptor interacting protein (RIP)-1. RIP-1 binding to IKKγ is thereby disturbed and NFκB is retained in the cytoplasm, also inhibiting TNFR-induced signaling [45,148,149]. Additionally, decreased signaling, induced via other receptors, was found to involve the inhibition of NFκB by A20. In line with the anti-inflammatory role of A20, expression of IL-1β, TNFα, C-reactive protein (CRP), and other inflammatory mediators, as well as lipid peroxidation, were downregulated as found in humans, mice and chicken due to altered zinc homeostasis [148,150–153].

Another zinc-finger protein which plays a role in homeostatic zinc signaling is peroxisome proliferator-activated receptor (PPAR)α. Similar to A20, PPARα expression was induced by zinc. PPARα alters the binding of NFκB to DNA and thereby abrogates the induction of pro-inflammatory cytokines and adhesion molecules [150].

5.3. Zinc Alters Hematopoiesis by Altering Intracellular Signaling

In mice, acute phase reaction was paralleled by transient serum hypozincemia and an increase in the leukocyte count in the blood [63]. Moreover, long-term dietary-induced zinc deficiency affected hematopoiesis in mice, causing a shift in the ratio of leukocyte subsets towards innate myeloid immune cells [154]. In humans, T cells were clearly diminished during zinc deficiency. A prioritizing of cells from the myeloid lineage (monocytes and granulocytes), at the cost of lymphoid cells (especially the T cell compartment), if zinc supply is limited, has been hypothesized in humans as well, however this remains to be proven.

Decreased intracellular zinc levels were shown to be necessary for the differentiation of monocytes and dendritic cells and were suggested to play a role in granulopoiesis [80,100]. In dendritic cells, LPS stimulation caused expression of ZIP6 and ZIP10 as well as of ZnT-1, ZnT-4, and ZnT-6 via pathways involving TRIF, resulting in an altered cellular zinc homeostasis [100]. Alteration in TRIF signaling by the zinc status of a cell has also been described for LPS stimulated macrophages [97].

One centrally important molecule regulating differentiation in various cell types is STAT3. Recent studies revealed that phosphorylation, and thereby activation of this signaling molecule, was altered depending on the zinc status of a cell, while total protein concentration remained unaltered [155]. In myeloid cells, TLR3 and NLPR3 inflammasome-triggering activated the janus kinase (JAK)-STAT3 as well as the MAPK pathway. Pre-existing zinc deficiency augmented the effect of IL-6-or IL-1-induced JAK-STAT3 signaling. When zinc deficient cells were reconstituted with zinc prior

FOI 26-3132 - Document 5

to stimulation, signaling was normalized [142]. STAT3 is phosphorylated by SHP1/2, known to be zinc-dependent PTP [156]. Zinc chloride increased transient STAT3 Tyr705 phosphorylation in murine hematopoietic stem cells, maintaining pluripotency and self-renewal of the stem cells, even replacing leukemia inhibitory factor (LIF), which is normally essential for this process. After zinc incubation, expression of pluripotency genes was increased and expression of differentiation-associated genes was inhibited. Zinc was able to prevent retinoic acid-induced differentiation, at least for a restricted time-frame [143].

Zinc deficiency caused B cell proliferation via increased STAT3 phosphorylation [145]. However, increased STAT3 phosphorylation was noted when murine embryonic stem cells were incubated with zinc chloride, suggesting that cell type specific effects exist [157]. In addition, the timing was different between the studies, which might help to explain the differences. In a very recent study, zinc was shown to alter STAT3 phosphorylation in neuronal cells via redox-mediated mechanisms. Interestingly, phosphorylation patterns of STAT3 residues were different when nuclear and cytoplasmic fractions were compared [155]. Whether this effect can be extrapolated from neuronal cells to immune cells remains to be tested. However, redox status and zinc metabolism are highly intertwined, so that a similar mechanism is highly likely. Finally, STAT3 was also suggested to be decisive for the switch from regular to demand-adapted emergency myelopoiesis during inflammatory diseases such as sepsis [158]. However, the association between disease-related serum hypozincemia, STAT3 activity and emergency differentiation needs to be proven.

Maturation and differentiation of T cells in the thymus is affected greatly by zinc availability. It has long been shown that the activity of thymulin, an essential factor for T cell development, is severely disturbed during zinc deficiency. This results in disturbed suppressor function, allogenic cytotoxicity and IL-2 production [159]. In addition, zinc deficiency seems to decrease the expression of anti-apoptotic factors such as B cell lymphoma (Bcl)-2 and Bcl-xL by T cells. In connection with the increased levels of pro-apoptotic factors detected in serum during zinc deficiency, this explains the severe reduction of T cell numbers [160]. Furthermore, zinc was shown to affect caspase activity. The cell cycle regulator p21 was cleaved by caspase 3 during zinc deficiency, activating cyclin-dependent kinase 2 (CDK2). Consequently, cells entered prematurely into S-phase and were subjected to apoptosis [92,161]. In addition to its intra-thymic activity, thymulin affects proliferation and activation of T cells in blood and tissue [162]. As zinc supplementation was able to restore thymulin activity in the thymus as well as in blood and tissue in humans and mice suffering from mild zinc deficiency, the reversibility of defects arising from zinc deficiency is probable and therapeutic zinc application should therefore be beneficial [163–165]. Interestingly, expression of MT-1 and calprotectin is increased in various cell types during differentiation. As both are important intracellular zinc binding proteins, this could increase the amount of zinc immediately available for signaling processes [80,166]. The latter might explain the manifold stronger reaction of mature cells to stimulation compared to immature cells, as the magnitude of the fast zinc signal is most probably much higher due to high MT levels.

5.4. Zinc, Smad Signaling and the Development of Regulatory T Cells

The natural importance and therapeutic benefits of regulatory T cells (Tregs) in preventing auto-immune diseases and transplant rejection is becoming more and more accepted [167]. One important trigger for Treg development is TGF-β-induced Smad 2/3 signaling, which induces expression of the transcription factor forkhead-box-protein p3 (FoxP3), which is essential for lineage commitment. Zinc supplementation augmented TGF-β-induced Smad 2/3 signaling and thereby FoxP3 expression [168]. Proteasomal degradation of FoxP3 involves the histone deacetylase Sirtuin-1 (Sirt1), which is inhibited by zinc as was shown for other histone deacetylases as well [72,169]. Another important, zinc dependent molecule in Treg cell development is interferon regulatory factor (IRF)-1, as it is able to repress FoxP3 activity [170]. Zinc supplementation dampened IRF-1 activity, increasing tolerance.

FOI 26-3132 - Document 5

In addition to its role in Treg development, zinc also affected Treg function. Here, CK2-mediated activation of ZIP7 was of central importance [171]. The transporter ZIP7 is located in the endoplasmic reticulum and responsible for the release of zinc into the cytosol. Phosphorylation of ZIP7 by casein kinase II (CK2) activated it, causing a zinc flux, which was suggested to cause activation of signaling pathways, resulting in cell proliferation and migration [171]. Thus, zinc was able to induce and stabilize Treg cells in this study, underlining its therapeutic importance.

Rice et al. investigated MT expression and efficiency of signal transduction in Type 1 regulatory T cells (Tr1), another recently described member of the family of Treg cells. Intracellular zinc and MT expression were increased during differentiation of naïve T cells into Tr1 cells after six days [172]. When IL-27 was also added, zinc increases and MT-1 expression was augmented. In addition, cells with higher MT levels more efficiently transduced a ROS signal into a zinc signal, as monitored via p38 MAPK signaling. MT overexpression was observed during chronic inflammatory diseases and cancer. The correlation of MT expression with efficiency of signal transductions might explain the high reactivity of cells during chronic zinc deficiency [172]. Whether this explains hyperinflammation, observed during sepsis-induced augmented serum hypozincemia, needs to be explored. Calprotectin is another zinc binding protein that is elevated during severe inflammatory disease and serum hypozincemia [63]. In contrast to MT, expression of calprotectin was recently shown to be induced by zinc deficiency, similarly to what has been described for pro-inflammatory cytokines [66,173].

5.5. PMA-Induced NET Formation in Granulocytes

A representative example illustrating nicely the importance of zinc in regulating cell signaling is the formation of NETs by granulocytes. ROS production in neutrophils via NADPH oxidase is an important prerequisite for NET formation. Both zinc deficiency as well as excess have been shown to inhibit superoxide anion production [174] and thereby the formation of NETs. When zinc was chelated in granulocytic cells, the PMA-induced increase in intracellular zinc was abolished, as well as NET formation [83], showing that the assumption of a tight zinc and redox metabolism interrelationship holds true for granulocytes and their functions. Whether other pathways are involved in NET induction remains to be investigated.

5.6. TLR4-Induced Signaling: A Great Example of Fine-Tuning of Signaling Pathways by Zinc

Triggering of TLR4 at the surface of myeloid cells by LPS or other ligands activates several signaling pathways as illustrated in Figure 2. This is a great example to illustrate how zinc flux and homeostatic zinc signals are able to fine tune cell signaling. This example also shows how long-term changes in extracellular zinc resulted in different effects to those from acute alterations.

LPS binding to TLR4 caused a zinc flux, which subsequently inhibited dephosphorylation of MAPK and supported phosphorylation of IKKα/β so that NFκB translocated into the nucleus and activated expression of pro-inflammatory cytokines [94]. When zinc signals were prevented using chelators such as TPEN (*N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine), activation of kinases ERK1/2, IKKβ, MKK3/6 and IκB was abrogated, as shown for murine macrophages. Zinc chelation blocked the destruction of IRAK1 causing its accumulation in the cytosol. In contrast, IRAK1 phosphorylation and ubiquitination were not affected shortly after LPS-triggering. This might be due to the inhibition of zinc-dependent MMPs, which are probably responsible for IRAK1 destruction during adequate zinc conditions. IRAK1 is responsible for the degradation of NFκB inhibitors by IKK. Therefore, NFκB did not translocate to the nucleus. Furthermore, ERK signaling was disturbed when zinc was chelated, which might be associated to the effect of zinc on PTP and PTK activities as described earlier [175,176].

The MAPK and NFκB pathways described above are all induced by TLR4 ligation via myeloid differentiation marker (MyD)88. In addition, MyD88-independent/Toll-interleukin-1 receptor (TIR) domain-containing adaptor-inducing interferon (TRIF)-dependent pathways involving TRIF-related

FOI 26-3132 - Document 5

adaptor molecule (TRAM), TRIF and interferon regulatory factor (IRF)-3, are also activated. In contrast to pro-inflammatory mediators, activated via MAPK and NFκB, IFNβ expression is triggered here, which functions in an autocrine fashion and activates the JAK-STAT pathway as well as a delayed translocation of NFκB to the nucleus after binding to the IFNR on the cell surface. This upregulates CD40, CD80, and CD86 expression enabling the cell to interact with T cells [97,177].

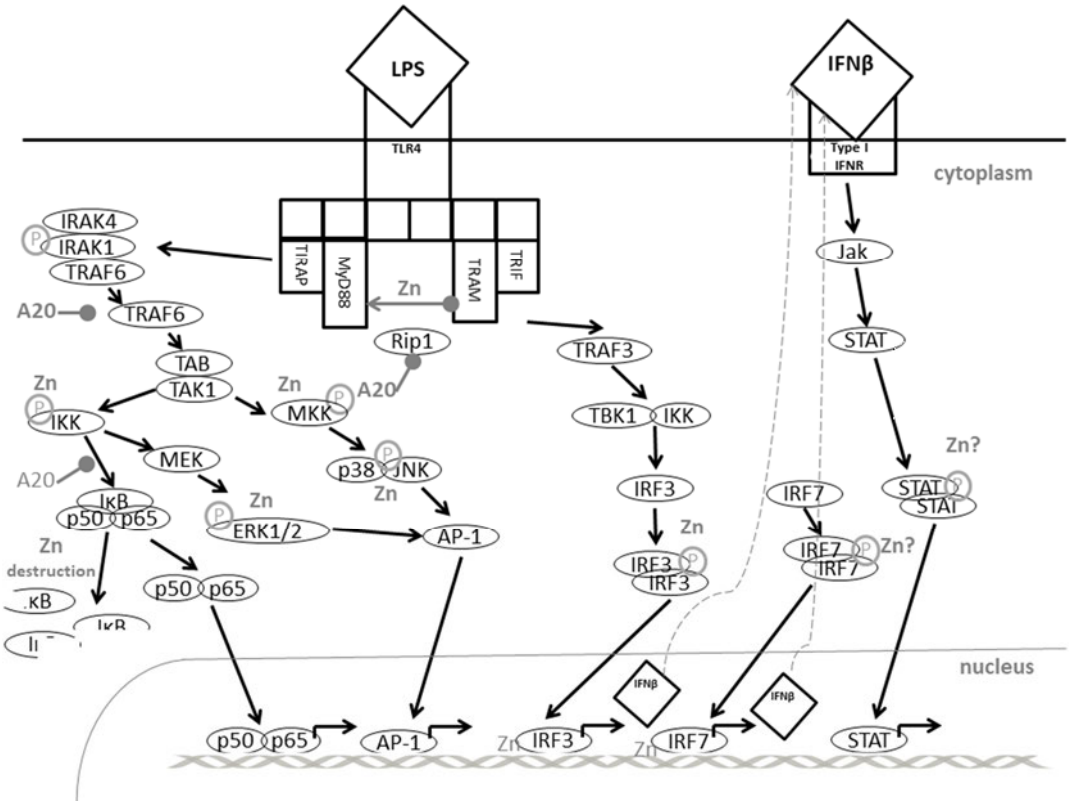


Figure 2. Zinc in Toll like receptor 4-induced signaling. For explanation, see text. Abbreviations: A20: zinc finger protein; AP-1: Activator protein 1; ERK: extracellular Signal-regulated Kinase; IFN: interferon; IRAK: Interleukin-1 receptor-associated kinase; IκB: Inhibitor of NFκB; IKK: IκB kinase; IRF: interferon related factor; JAK: JNK janus kinase; JNK: c-Jun *N*-terminal Kinase; LPS: Lipopolysaccharide; MAPK: mitogen activated protein kinases MEK: MAPK/Erk kinase; MKK: MAPK kinase; MKP: MAPK phosphatase; MyD88: Myeloid differentiation primary response gene; NFkB: nuclear factor (NF)κB; PI3K: phosphatidyl-inositol-3-phosphate; RIP: receptor interacting protein; STAT: Signal transducers and activators of transcription; TAB: TAK-binding protein; TAK: TGF β-activated kinase; TBK: Tank-binding kinase 1; TIRAP: toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TLR: Toll like receptor; TRAF: TNF receptor-associated factor; TRAM: TRIF-related adaptor molecule; TRIF: Toll-interleukin-1 receptor (TIR) domain-containing adaptor-inducing interferon. Altered from [97,176,178].

Furthermore, the inducible nitric oxide synthases (iNOS) is induced via the JAK-STAT pathway, resulting in NO production to support the anti-microbial reaction. During zinc deficiency, murine macrophages were shown to produce more NO after LPS stimulation than controls. Zinc deficiency also augmented LPS-induced expression of IFNγ, CD80 and CD86 in murine macrophages either bone marrow-derived, or from cell culture, while expression of IL-1β, IL-6 and IL-10 was decreased. All effects were normalized when zinc deficient cells were reconstituted with zinc prior to stimulation. Here, analyses were performed a few hours after LPS stimulation, representing homeostatic zinc signals. As incubation of cells with a zinc chelator after LPS-stimulation still had the

FOI 26-3132 - Document 5

same effects, one can conclude that effects on MyD88-independent pathways are not associated with the zinc flux, but rather by the homeostatic zinc signal [97].

The delayed favoring of the MyD88-independent pathways is paralleled by inhibition of parts of the MyD88-dependent pathways as well. Here, ubiquitinated TRAF6 activates IKK and MKK via the kinase TAK1. However, as mentioned earlier, ubiquitin residues are removed from TRAF6 by A20, highly expressed due to the homeostatic zinc signal, resulting in suppression of gene expression [148,179,180]. In addition to zinc, pro-inflammatory cytokines are known inducers for A20 in macrophages, possibly representing a feedback-mechanism to prevent hyperinflammatory reactions [181]. This fits well with the deregulated inflammatory response seen during chronic and severe serum hypozincemia, as observed during sepsis [63,65]. Finally, a role of zinc in TLR4-triggered signaling via zinc's effect on cyclic nucleotides was suggested. In this connection, elevated cGMP could be responsible for increased Raf-1 phosphorylation, which inactivates this MAPK and also prevents NFκB activation and translocation. Signaling induced via other TLR might be regulated in a similar manner. Zinc-dependent regulation of TRIF/IRF/IFNβ signaling was observed as well when cells were stimulated with TLR3- or TLR7-specific agents [97].

Recently, a first approach to analyzing the zinc-dependents of the proteome was published. Here, macrophages were incubated with either zinc oxide nanoparticles or zinc acetate in equivalent doses. After at least one day, the proteome of the cells was analyzed using a 2D gel electrophoresis technique. The amount of several signaling molecules was altered by zinc incubation. Most interestingly, MyD88 protein levels were altered due to incubation with the nanoparticles or the zinc acetate [182]. Unfortunately, there is so far no information as to whether this is due to increased expression or decreased degradation of the protein and tests of MyD88 function were also not included, thus more analyses are needed. However, the results of this study suggest new targets that are probably affected by zinc signals and cellular zinc homeostasis.

Altogether, this is not only a great example of fine-tuning of signaling by zinc but also underlines the complexity of zinc's role in intracellular signal transduction.

5.7. Zinc Levels Regulate Fc Receptor-Induced Signaling

Instead of recognizing PAMPs, some immune cells are equipped with receptors for immunoglobulins (Ig) to detect immune complexes or opsonized pathogens. One example is the Fcε receptor (FcεR) on mast cells, whereby ligand binding causes degranulation and thereby the release of anti-microbial agents. Those granules are also known to contain high amounts of zinc, released during degranulation, probably causing zinc intoxication of pathogens in close proximity to the immune cell. The role of zinc in mast cells and their signaling has been poorly investigated, but some data indicate that triggering the FcεR causes a zinc wave via release of zinc from the perinuclear area, such as from the endoplasmic reticulum (ER) [96,183]. Thereby, PKC was transported to the plasma membrane, ERK1/2 and JNK1/2 were phosphorylated and NFκB was activated and translocated to the nucleus, inducing expression of pro-inflammatory cytokines [96,184]. When the zinc wave was prevented by incubation of the cells with TPEN, signaling and gene induction were blocked. In line with this, signaling was prolonged when zinc was added to the cells. In addition, zinc seems to be important for fusion of the granules with the plasma membrane, thus enabling degranulation [184]. Zinc chelation inhibited degranulation. Within these studies, two pools of zinc were identified by using different zinc probes: the larger pool of granular zinc and the smaller, variable pool of cytoplasmic zinc. This emphasizes that individual probes can vary in their intracellular location, also depending on the cell type and its activation state [71,185,186].

5.8. Killing Activity of NK Cells Varies with Zinc Availability

NK cells are able to destroy transformed or infected cells, largely due to recognition of changes in major histocompatibility complex MHC-I composition that are caused by the degeneration or the pathogen. In contrast, regular cells are protected from killing by NK cells as long as they carry

FOI 26-3132 - Document 5

surface MHC-I, which is bound by the killer cell inhibitory receptor (KIR) on NK cells [187]. To form the so-called NK cell synapse, KIR multimerization is essential, and was shown to be zinc-dependent [87,89]. Unfortunately, there are no data on complex formation during zinc deficiency, but a disturbance in zinc homeostasis would explain why killing is reduced if zinc supply is limited and increased if additional zinc is supplied [88]. Some recent data suggest a zinc signal after NK cell activation with IL-1β [188]. IL-1R signaling is known to be affected by zinc as will be described later. Because NK cells carry a variety of surface receptors containing tyrosine phosphorylation sites, including immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based activation motif (ITAM), a role for zinc in signal transduction is likely, and this possibility should be tested.

5.9. The Role of Zinc in IL-2-Induced Signaling in T Cells

When IL-2 binds to its receptor, signal transduction via Jak1 and Jak3 is initiated and the adaptor Shc is recruited. Oligomerization of the receptor is essential for its function, thus the IL-2R subunits are found in lipid rafts. Subsequent to IL-2 stimulation, three different pathways can be activated, centering around PI3K/Akt, STAT5 and MEK1/2-ERK1/2. IL-2 is known to induce proliferation and development of effector functions in T cells, inducing anti-apoptotic and cell-cycle related genes as well as certain cytokines and lineage decisive factors [189].

Similar to the effects of other growth-promoting factors, binding of IL-2 to its receptor induces a zinc flux. Here, zinc is released from lysosomes and zincosomes, which was linked to ERK1/2 and Akt activation, but did not affect STAT5. In this context, Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is of importance, as it degrades PI3K-produced PI(3, 4, 5)P3, which subsequently activates Akt. The zinc IC_{50} of PTEN was found to be 0.59 nM, which would allow its blockade by zinc flux, explaining activation of Akt [71,135].

5.10. Zinc in T Cell Receptor Signaling

The major function of the T cell receptor (TCR) is the specific recognition of a certain antigen, presented by an MHC molecule. As the TCR lacks intrinsic kinase activity, the CD3-complex, responsible for signal transduction, needs to be recruited and non-covalently bound to form the functional receptor. PTKs, involved in the subsequent highly organized signaling cascade include members from Src, Syk, Csk (C-terminal Src kinase) and Tec families. In addition, certain adapter proteins and effector enzymes are involved, as illustrated in Figure 3. Activation of the signaling molecules via phosphorylation by PTKs can be rapidly reversed, as PTPs have a higher capacity than the PTKs. Genes expressed after TCR triggering are dependent upon the stimulus and co-stimuli characteristic of a certain T cell subsets, causing proliferation and differentiation [109].

The importance of zinc as an ionic signaling molecule after TCR triggering has been investigated in various studies. A zinc flux was measured within less than one minute after ligand binding to the TCR. As silencing of ZIP6, located in the plasma membrane, prevented this fast zinc signal, the origin of the zinc seems to be extracellular [190]. When extracellular zinc was depleted from the media, no fast zinc signal occurred after TCR triggering, supporting this hypothesis. Subsequent to its fast increase, zinc is compartmentalized into a subsynaptic region, which is quite different from the calcium wave, which originates from intracellular stores and spreads rapidly throughout the cell [191]. However, in addition to extracellular zinc, the fast zinc signal might also be caused by ZIP8-mediated release of zinc from lysosomes, recruited to the TCR complex after stimulation [101]. In response to certain environmental conditions, basal expression of MTs was increased. This rendered more releasable zinc available for subsequent stimulation. Consequently, lower levels of MTs observed during prolonged zinc deficiency could explain the lower response of T cells to antigen, as less zinc for a zinc flux would be immediately available.

FOI 26-3132 - Document 5

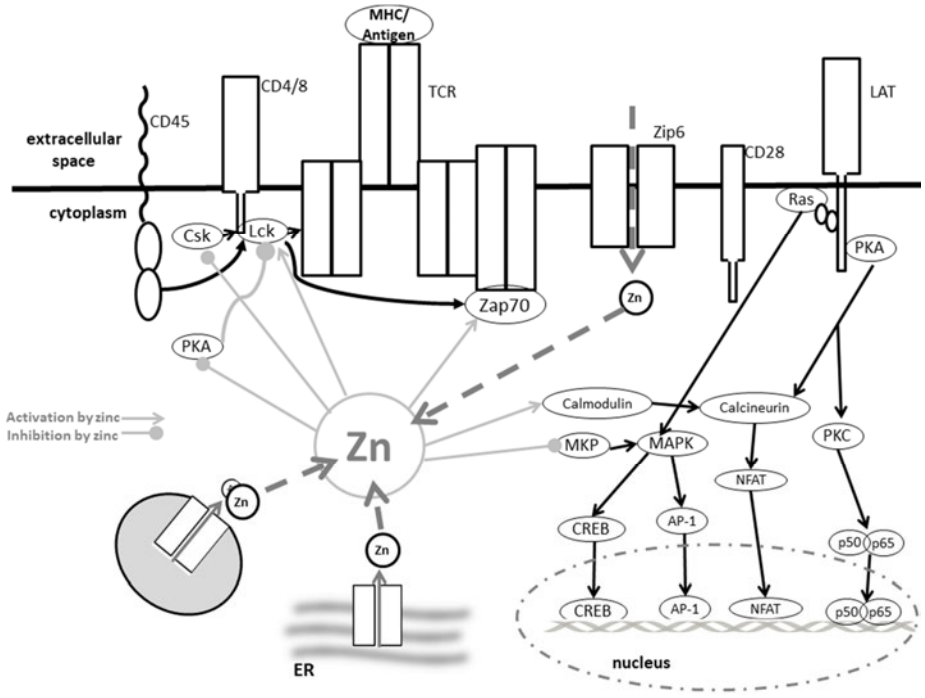


Figure 3. Zinc in TCR-induced signaling. For explanation, see text. Abbreviations: AP-1: Activator protein 1; CREB: cAMP response element-binding protein; Csk: C-terminal Src kinase; ER: endoplasmic reticulum; Lck: lymphocyte-specific protein tyrosine kinase; LAT: linker for activation of T cells; MAPK: mitogen activated protein kinases; MHC: major histocompatibility complex; MKP: MAP kinase phosphatase; NFAT: Nuclear factor of activated T cells; p50/p56: nuclear factor NF-kappa-B subunit p50/p65; PKA: protein kinase A; PKC: protein kinase C; Ras: Rat sarcoma; TCR: T cell receptor; ZAP: z-chain-associated protein kinase; Zip: Zrt-like, Irt-like proteins. Modified after [109,170].

Targets of this zinc flux have been found within several stages of signal transduction after receptor triggering. As has been described above, the TCR alone is not able to activate a signaling cascade. Therefore, CD4 brings the Src kinase Lck (lymphocyte-specific protein tyrosine kinase) into proximity with the TCR, thus enabling tyrosine phosphorylation of signaling molecules, including ZAP70 and CD3ζ. Zinc facilitated the binding of Lck to CD4, as it did to CD8, wherefore it is of central importance for complex assembly and the initiation of the signaling process [192].

Not only recruitment, but also activity of Lck, were affected by zinc, as it enabled Lck homodimerization by stabilizing the SH3 domain [193]. Lck activity can additionally be inhibited by phosphorylation of tyrosine Y505, regulated by the PTP CD45 and the kinase Csk [194], or activated by phosphorylation of Y394, which is dephosphorylated by PTPN22. A direct effect of zinc on the activity of phosphatases and kinases was described [195]. Zinc supplementation of T cell lysates did indeed result in dose-dependent decrease of total phosphatase activity [71]. In support of this, Csk is inhibited by zinc [196], as probably is PTPN22. Moreover, recruitment of PTPN22 is regulated by Csk [102]. A direct effect of zinc on CD45 activity has been found in vitro, when zinc was supplemented in high doses, and this remains to be investigated in more detail [103]. As Lck is responsible for the phosphorylation of ZAP70, which then further activates signaling molecules such as MAPK, abrogation of the initial zinc flux can have far reaching consequences [71,197,198].

Further downstream, zinc might also affect transcription factor binding. One important target is NFAT, responsible for IL-2 expression, but retained in the cytoplasm of resting T cells due to constitutive phosphorylation. After TCR triggering, it is dephosphorylated by CN, serine/threonine phosphatase [199]. As physiological concentrations of zinc between 10 nM and 10 µM were able to

FOI 26-3132 - Document 5

inhibit CN activity, NFAT translocation might also be affected by the zinc flux. However, no direct evidence is available and, as the zinc signal seems to stay local, i.e., close to the receptor, this needs to be further investigated. In addition, regulation of CN activity is rather complex. CN itself is constitutively inactivated by phosphorylation. Here, phosphatidyl-inositol-3-kinase (PI3K) is responsible. PI3K activity is also zinc sensitive. Zinc supported enzymatic activity via effects on Akt and PTEN explored in detail for IL-2-induced pathways [135]. If effects are similar for TCR-induced signaling, needs to be explored.

In this regard, consequences of homeostatic zinc signals will be described, as they also affect CN activity. TCR triggering causes upregulation of ZIP8 expression. Therefore, zinc is released from the lysosomes causing an increase in intracellular zinc, when the zinc flux has probably already vanished. This homeostatic zinc signal has been connected to a blockade of CN activity which results is transcription of IFNγ [101]. Another consequence of the homeostatic zinc signal is augmented PKC activity due to facilitated binding of phorbol esters and higher affinity to cytoskeleton and plasma membrane [119]. As a final note on the importance of zinc for TCR-triggered T cell response, the activation threshold for stimulation was lowered when additional zinc was supplemented to the cells. Thus, even suboptimal antigen conditions caused T cell proliferation [102], which might be interesting for optimizing vaccination. As a note, calcium influx was unaltered, when zinc conditions were changed.

When T cells are activated, MT expression increases, which was associated to decreased intracellular zinc and ROS levels and a sustained proliferation of the T cells [101]. Thus, also for TCR-induced signaling, effects of zinc further downstream are likely. As the zinc flux increased MT expression in the T cells, not only immediate signaling pathways were affected, but T cell proliferation and survival were supported in the long term [200]. In addition, TCR ligation was also followed by ROS generation, known to provide zinc from protein for signaling regulation [201]. Triggering of TCR led not only to increased activity, but also to an increased expression of ZIP6 [202]. The influx of extracellular zinc inhibited the recruitment of SHP-1. As SHP-1 is a negative regulator of TCR-signaling, this might function as a feedback mechanism to end TCR-mediated signaling [102]. The lower recruitment of SHP-1 might either be due to changes in its conformation or the serine phosphorylation of Lck, which prevents SHP-1 binding.

5.11. The Role of Zinc in IL-1 Receptor Signaling in T Cells

IL-2 functions as a majorly proliferative agent for T cells, whereas other cytokines, such as IL-1 also induce the expression of inflammation-related genes such as IFN-γ, in addition to supporting proliferation. After IL-1R triggering, IL-1 receptor-associated kinase (IRAK) is activated, causing NFκB translocation to the nucleus, culminating in gene activation. Here, zinc supplementation of T cells was shown to decrease IRAK activity, similarly to its decreasing TLR4-induced signaling pathways, resulting in repression of the memory Th17 response [97,203]. It has been reported that IL-1 binding causes a zinc signal [204]. MyD88 is another central signaling molecule of IL-1-induced signaling, and a role for zinc in this pathway has been described [97]. Effects have not been explored for the IL-1R, yet.

The last paragraphs underline the complexity of zinc's effect on T cell development, lineage decision and activity. As effects were described for each pathway individually, this is an oversimplification of the in vivo situation. In addition, effects of for example zinc supplementation and zinc deficiency on the outcome of the investigated T cells will also depend on other environmental factors and the cellular environment, rendering prediction of an overall effect impossible.

5.12. The Role of Zinc Signaling in B Cells

When B cells are mentioned, antigen recognition and antibody production come to mind. Antigen recognition, and thereby choice and activation of a certain B cell clone, is mediated via the B cell receptor (BCR). BCR activates almost the identical intracellular pathways to TCR. These pathways focus, amongst others, on the same kinases (PKC, MAPK) and transcription factors (NFAT, NFκB) [108].

FOI 26-3132 - Document 5

Surprisingly, the correlation between zinc status and the proliferation and overall functions of B cells was much weaker than for T cells, as was shown by investigations in mice [205,206]. A considerable difference was found between the effects of acute and chronic zinc deficiency: While acute zinc deficiency caused a reduction in overall B cell numbers, this was far less pronounced during chronic zinc deficiency [207]. Recently, the regulation of ZIP10 expression by STAT3 and STAT5 was revealed and associated with suppression of apoptosis in human B cell lymphoma, suggesting some effects of homeostatic zinc signals on the development and survival of B cells.

Generally, binding of antigen alone is not sufficient to activate the complete maturation of a B cell into an antibody-producing plasma cell. Recent studies indicate that ligation of the B cell-activating factor receptor (BAFFR), TLRs and CD40, synergize with the BCR to define the repertoire of the mature B cell [208]. Additionally, co-stimulation by T cells is required. As T cell numbers and activation are strongly compromised during zinc deficiency, zinc was first suggested to play an indirect role. This was in line with the observation that especially T cell-dependent antibody production was affected [209]. However, antibody production per cell was largely constant under zinc deficient conditions [210]. To circumscribe effects due to alteration of T cell numbers and functions, Hojyo et al., in an elegant study, blocked the zinc signal in B cells, while not affecting the T cell compartment [211]. The group identified ZIP10 as being responsible for zinc signals in antigen-presenting cells, including B cells. To specifically knock out ZIP10 in antigen-presenting cells (APC), the ZIP10 gene was conditionally ablated by being put under the control of the invariant chain promotor. The invariant chain gene is constitutively activated in APC, but not in other cells including T cells. Interestingly, BCR-induced signaling was severely disturbed in cells from ZIP10 knockout mice and production of specific immunoglobulin (Ig)M and IgG was strongly decreased, which suggest that zinc indeed plays an important role in BCR-induced signaling. Interaction with T cells usually induces the germinal center (GC) reaction necessary for Ig class switch in B cells. Formation of GC B cells was also reduced in cells from ZIP10 knockout mice. BCR-crosslinking-induced proliferation was diminished, although hyperactivation of ERK, AKT and NFκB was observed and activity of SYK and LYN was increased. Only CD45 activity was less than half in cells from ZIP10 knockout mice compared to ZIP10 expressing controls [211]. In general, those observations are in line with what has been observed in zinc-deficient animals, suggesting that not only indirect effects via the disturbed T cell compartment are responsible for the defect in B cell response. Of note is that TLR-induced signaling was not affected by ZIP10 knockout. As the origin of zinc for TLR-triggered zinc signals is largely protein and organelle-based, this is also in line with previous findings [94,98,212].

In addition to BCR-induced signaling, pathways in B cells affected by zinc included those centered on STAT3 and STAT6, activated for example by IL-6 or IL4, respectively. Here, in vitro studies revealed that IL-4-induced STAT6 and IL-6-induced STAT3 phosphorylation were decreased during zinc deficiency [145]. As the IL-4-induced pathway is essential for the immunoglobulin class switch to IgE, this might explain why susceptibility towards especially parasite infections is increased under low zinc conditions [145]. IL-6 is essential for the induction of terminal B cell development into plasma cells [213], providing an additional reason for the decrease in antibodies during zinc deficiency. On the other hand, IL-6 is known to induce the production of autoantibodies if plasma levels are highly elevated. Thus, the association of IL-6, STAT3 phosphorylation, and (auto-) antibody production needs to be investigated more closely.

Furthermore, zinc deficiency was shown to increase apoptosis in B cells during early developmental stages, similar to what was described for T cells [214]. Fewer naïve B cells were found during zinc deficiency, while numbers for precursor B cells and cycling pro-B cells were not largely affected. While the response to known antigens was still efficient, reaction to neoantigens by B cells declined during zinc deficiency. This might be one reason for the decreased efficiency of vaccination in the elderly, who are known to be at least marginally zinc deficient in most cases [215,216]. Along with this, zinc uptake into cells through the zinc transporter ZIP10 was essential for early B cell development, increasing cell survival [217]. Others found that the effect of zinc on apoptosis via

FOI 26-3132 - Document 5

caspase activity depends on the dosage: treatment of Burkitt Lymphoma cells with up to 50 μM of zinc inhibited caspase 3 activation and apoptosis, whereas higher concentrations up to 100 μM rather induced programmed cell death via caspase 3 [218].

Homeostatic zinc signals affected various stages in apoptotic signaling cascades, including production and activity of members of the Bcl/Bax family [214]. In addition, DNA fragmentation was decreased when sufficient zinc was available, as the endonuclease responsible for this process is calcium dependent but inhibited by zinc. Furthermore, caspases 3, 6 and 8 were inhibited by zinc in a dose-dependent manner. IC_{50} for caspase 3 was for example 10 nM zinc, which is within the physiological intracellular range [137,219]. These three caspases are known mediators within the apoptotic pathway [220].

6. Zinc and Transcription Factors

In the early 1980s, zinc binding sites were found in *Xenopus laevis* transcription factor IIIA (TFIIIA) 24. This discovery offered new approaches to discover structural zinc binding sites in other species similar to those uncovered in *Xenopus laevis*. Thus, for the first time the typical zinc-finger structure was defined in TFIIIA and consisted of nine repetitive sequences containing cysteine (C) and histidine (H) residues. Since then, the discovery of zinc-binding sites dramatically increased within a short period, based on homology searches in sequence databases for the characteristic ligands in TFIIIA. Today, nearly 3000 proteins with zinc-binding motifs have been identified by bioinformatic approaches, making up ~10% of the proteins encoded within the human genome potentially regulated by zinc [43].

Regarding the human immune system, the innate and adaptive immune system are both regulated by zinc-finger-bearing transcription factors. Hence, a direct as well as an indirect role of zinc in altering intracellular signaling can be anticipated. During the development of innate immune cells, essential transcription factors such as GATA-4/-5/-6 and KLF-4/-5 are potentially zinc-regulated targets since they contain a zinc-finger domain [221,222]. Moreover, the promyelocytic leukemia zinc-finger is a critical transcription factor for iNKT cell development. In myelopoiesis, expression of the transcription factor PU.1 is decisive for lineage commitment [223]. With respect to the adaptive immune system, a multitude of zinc-finger-containing transcription factors have been identified. In T cell development, various transcription factors are important for the adequate differentiation into $CD4^+$ T cells, such as the zinc-finger transcription factors GATA-3 and Zbtb7b (Thpok, cKrox) [224,225]. Moreover, GATA-3 is essential for Th1 and Th2 subpopulation differentiation [226,227]. Generally, the zinc-finger-containing KLF family has an important function in immune cell differentiation, for example KLF-2/-3 in B cells, KLF-2/-13 in NKT cells, and KLF-2/-4/-10 in T cells [228]. Consequently, it is not surprising that Treg differentiation is driven, amongst other things, by zinc-dependent KLF-10 modulation [170].

Concerning the overall regulation of zinc homeostasis, numerous genes exhibiting metal response elements in their promotors are involved. These can be induced by binding of the metal-response element-binding transcription factor (MTF)-1 that itself is regulated by the surrounding available free zinc concentration. Thus, MTF-1 acts as an intracellular zinc sensor, leading to the suggestion that feedback mechanisms may exist [229]. The field of zinc-regulated transcription factors is still under investigation and offers great opportunities for further research to better understand modulation of the immune function due to zinc supplementation or zinc deficiency, respectively. Regulation of transcription factors by zinc homeostasis is a good example of how a single element such as zinc can balance the immune response and therefore acts as a "gatekeeper".

7. Zinc, Epigenetics, and Immunity

Over 75 years ago, Waddington chose the term "epigenetics" to describe changes to DNA other than in its sequence that were able to alter gene expression [230]. Those non-DNA-encoded changes include mainly DNA methylation (methylation of cytosine bases) and modification of histone tails by adding chemical groups such as one or more acetyl, methyl, or sumoyl residues. Alterations cause

FOI 26-3132 - Document 5

nucleosome re-positioning and expose or block binding sites for transcription factors or suppressors, thereby promoting or inhibiting the expression of adjacent genes [231]. Non-coding RNAs and transcription factor regulatory networks also count as epigenetic mechanisms. During recent years, the field of nutritional epigenetics emerged, covering the effects, amongst others, of nutritional elements such as zinc, but also barium, calcium, chromium, manganese, magnesium, iron, selenium, sodium, molybdenum, phosphorus, potassium and sulfur on the epigenome [232,233].

The finding that the establishment of the epigenome starts as early as during oocyte development, clearly underlines the importance of a well-balanced nutritional and mineral supply during pregnancy and breast-feeding. Zinc supply influences health and development from early in life, into old age [234]. In addition, lifestyle-dependent changes in the epigenome can occur throughout life. Dysregulations within the epigenome have been observed to accumulate in the elderly [235]. Epigenetic changes can even be carried over to the next generation, as has been shown by experiments with rats [236]. Various environmental factors, including: (1) individual characteristics such as age, gender and genetic predisposition; (2) lifestyle-related factors including the amount of exercise, smoking, and stress; and (3) nutrient-related factors such as dose, route, duration of the exposure as well as the presence and variety of elements, affect the epigenome and are summed up as "interactome". However, the interactome is difficult to estimate, as already drinking water and soil might contain different amounts of each trace element depending on the region [233]. Dysplasia and disease originating from all developmental stages, including diabetes, multiple sclerosis and other mainly auto-immune and neurological diseases have so far been connected to epigenetic abnormalities, caused by nutritional dysbalance, as will be exemplified later regarding zinc [233,237,238].

As indicated earlier, zinc is an important co-factor for enzymes from all classes of enzymes. This includes epigenetically-active enzymes such as class I, II, and IV histone deacetylases (HDACs) [239] and acetylases (HATs), DNA methyltransferases (DNMTs), histone methylases and methyl-binding proteins. Zinc deficiency has been associated with global DNA hypermethylation [240]. One explanation is the effect of zinc on activity of methionine synthase. This enzyme is responsible for the regeneration of methionine from homocysteine, providing substrates for DNA and histone methylation [241].

Disturbance of bone development during zinc deficiency has been shown to be epigenetically determined. Indirect effects of zinc on the epigenome include the multi-zinc-finger enzyme Trps1, named after the "Tricho-Rhino-Phalangeal syndrome", where it is deregulated. During mitosis Trps1 regulates histone deacetylation in a zinc-dependent manner. In addition, it increases the activity of HDAC1 and HDAC2, leading to histone H3 hyperacetylation if *Trps1* is non-functional. As a consequence, prometaphase cells accumulate due to impaired HP1 binding and chromatin condensation in Trps1-deficient chondrocytes [242]. Trps1 might be involved in regulation of mitosis in T cells, which needs to be explored in more detail and would offer another explanation for the dependents of hematopoiesis on zinc homeostasis.

As is true for most trace elements, zinc deficiency during pregnancy was associated with abnormalities in development of the embryo and poor health of the newborns, amongst others due to disturbances in immune cell development [243,244]. Here, various studies revealed that effect on development depends on the stage at which zinc deficiency was present. Regular development of the oocytes was paralleled by increasing methylation of their chromatin. Moreover, zinc was required to complete meiosis, as was shown for oocyte development in vitro and in vivo [236]. If the oocyte was zinc deficient after fertilization, meiosis failed due to disorganized chromatin, which should also be investigated in immune cell development. Various pathologies, such as chronic inflammatory diseases that appear later in life, have been associated with zinc deficiency during pregnancy. In addition to hypertension, autism and impaired learning, persistent immunodeficiency was observed for three subsequent generations in rats [245–248]. Interestingly, the regulation of genes, involved in zinc homeostasis was also affected by epigenetic mechanisms. Zinc-dependent epigenetic changes in the MT-2 gene in utero caused significantly higher MT-2 expression in the liver later in life as

FOI 26-3132 - Document 5

observed in mice [249]. Similarly, not only the function, but also the expression of zinc-finger proteins, including ZNF804A and ZNF326, was affected by the zinc status via epigenetic mechanisms [250,251]. As found during investigations in autistic patients, zinc deficiency during pregnancy caused epigenetic abnormalities in genes encoding MTF1, ZnT5, COMMD1 (COMM domain-containing protein 1), ERK1, TrkB (tyrosine-related kinase B), and ProSAP/Shank (proline-rich synapse-associated protein/SH3 and multiple ankyrin repeat domains) [252–255]. As those are important players regulating immune cell development and function, investigations on similar effects in immune cells are warranted.

Chronic inflammation, but increased susceptibility to infections are a typical symptom of aging. Amongst others, the accumulation of unusual epigenetic changes during aging was suggested to be responsible for altered activation of immune cells, causing those symptoms. Basal expression of inflammatory mediators such as IL-1β, IL-6 and TNFα was increased in vitro, largely due to changes in chromatin structure of the corresponding genes [66,185]. As the expression of zinc transporters was also altered via epigenetic modifications, this creates a self-reinforcing positive feed-back loop [256,257]. In particular, ZnT5, ZnT1, and ZIP6 expression was affected, causing a decline of zinc in the elderly [241]. The reversibility of epigenetic changes if zinc is supplemented needs to be explored.

8. Zinc Deficiency in the Context of Disease

Zinc status is strictly regulated by the zinc transporting proteins ZIP and ZnT. Malfunction of either ZIP or ZnT causes tremendous immunological impairment. Altered zinc homeostasis in the context of clinical manifestation of disease is summarized in Figure 4.

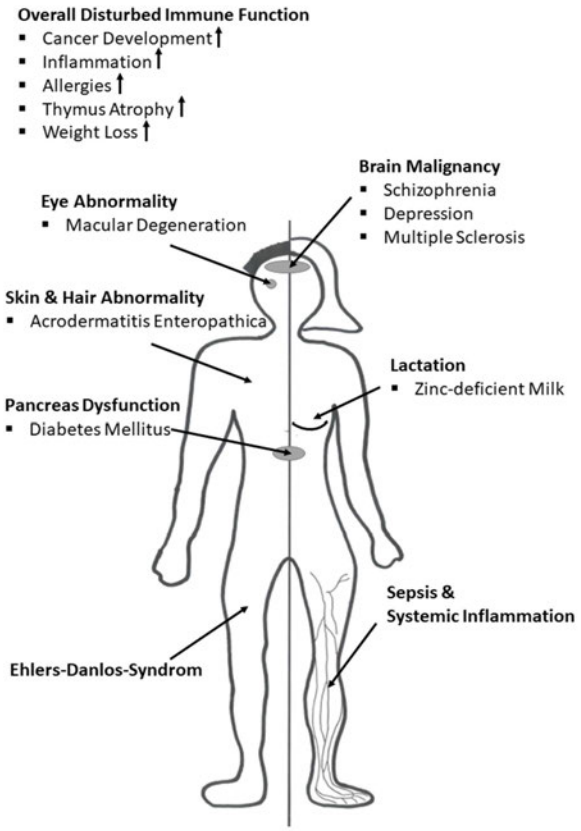


Figure 4. Influence of zinc and zinc deficiency on various organ systems as well as on the immune system. Zinc deficiency is causally associated with multiple immunological dysfunctions that lead to the manifestation of various diseases indicated in this figure. For a detailed explanation see the text. Modified after [5]. ↑ = upregulted/enhanced.

FOI 26-3132 - Document 5

8.1. Zinc and Acrodermatitis Enteropathica

Disruption of the ZIP4 gene was reported to impair early embryonic development in mice. This was due to malfunction of appropriate zinc transport into the developing embryo via the visceral yolk sac and to insufficient zinc uptake in the intestine, resulting in severe zinc deficiency. Similar effects of a non-functioning ZIP4 transporter and subsequent zinc deficiency were observed in humans suffering from Acrodermatitis Enteropathica (AE), as discussed above [47,258]. Patients suffering from AE showed several immunological alterations, such as thymus atrophy as well as decreased lymphocyte counts and lymphocyte function. This was not surprising, since the function of virtually all immune cells strictly depends on zinc homeostasis. Especially T cell function and maturation are highly zinc dependent, as described later in detail. Hence, AE patients have an increased susceptibility to infection, especially intercurrent bacterial, fungal, and other opportunistic infections are prevalent. Moreover, chronic diarrhea and lactose intolerance are also known, and without therapy AE is in general lethal. However, dietary zinc supplementation reversed the symptoms in both mice and humans [17,47,259]. T cell restricted malfunctions as well as abnormal chemotaxis were completely corrected by zinc supplementation.

8.2. Zinc in Cancer Development

The zinc transporter ZIP4 was shown to have an important role in cancer development. Microarray analysis identified steady state levels of ZIP4 mRNA to be highly upregulated in pancreatic cancer [260]. Biopsy analysis uncovered elevated ZIP4 mRNA expression in tumor samples as well as overaccumulation of ZIP4 protein [261]. Cell culture analysis determined that ZIP4 overexpression in human pancreatic cells increased zinc accumulation and cell proliferation respectively showing a connection between zinc status and cancer progression. Comparable results were obtained in experiments with mice. ZIP4 overexpression increased tumor weight as well as pathological fluid accumulation (ascites incidence). Additionally, increased zinc accumulation was apparent, as was a higher rate of cellular proliferation [262]. Knockdown of ZIP4 gene expression by siRNA inhibited tumor growth significantly and increased the survival rate of the mice [263]. Interestingly, increased ZIP4 expression was additionally found in hepatocellular carcinomas [264], emphasizing a potential role for ZIP4 in tumor development in general. At the molecular level, it was supposed that zinc transport via ZIP4 influenced cAMP response element-binding protein (CREB) activity, controlling numerous factors important in cellular proliferation [263,265,266]. Moreover, ZIP4 probably suppresses apoptosis and increases in vitro tumor cell migration [264].

In regard to breast cancer, the zinc transporters ZIP6, ZIP7 and ZIP10 were shown to be mainly involved in growth and invasive behavior. ZIP6, a member of the LIV-1 subfamily of ZIP proteins, was found to be estrogen-responsive and can therefore be used as biomarker for estrogen-receptor-positive cancers [267,268]. Additionally, luminal A-type breast cancer can be identified by ZIP6 expression [137]. Besides ZIP6, ZIP7 were highly abundant in breast cancer and were required for increasing the intracellular free zinc level [269,270]. ZIP10 expression was associated with breast cancer metastasis in lymph nodes and was strongly linked to invasive behavior [271]. Thus, ZIP6, ZIP7, and ZIP10 expression contributed to cancer cell survival and cancer progression [272,273]. At the molecular level, E-cadherin expression was modulated by ZIP6 as well as epidermal growth factor receptor (EGFR), Src, and insulin-like growth factor 1 receptor (IGF-1R) signaling molecules by ZIP7. Hence, cancer growth and invasive behavior is supported [274]. More recently, studies indicated a direct connection of phosphorylation of ZIP7 to be important for zinc efflux from intracellular zinc stores into the cytosol, leading to an elevated cytoplasmic free zinc level [98,275]. Consequently, signaling pathways such as mitogen-activated protein kinase (MAPK) signaling and tyrosine kinase activity were altered, leading to modulation of kinases and promoting aggressive breast cancer behavior. Amongst others, GFR, Src and IGF-1R signaling molecules were affected by zinc [269,276,277]. Cancer cells were highly apoptosis resistant due to an elevated intracellular zinc level, since caspase activity was found to be zinc-regulated [214]. Thus, killing of

FOI 26-3132 - Document 5

cancer cells by NK cells and cytotoxic T lymphocytes (CTL), respectively, was compromised. Moreover, a zinc-rich milieu induced Treg, impairing the anti-tumor response by inducing tolerance. Treg secreted high amounts of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β1 [278], constraining immunological anti-tumor defense.

8.3. Zinc and Schizophrenia

It was reported recently that nonsynonymous variants in both ZIP8 [279] and ZIP12 expression [280] were associated with schizophrenia. The brains of patients suffering from schizophrenia showed lower zinc concentration than healthy controls [281]. In general, ZIP12 has a conserved function in vertebrate nervous systems, being responsible for proper development. A correlation between a homozygous missense mutation in the *ZIP12* gene and the frequency of schizophrenia was detected and claimed as a possible explanation [37,282]. Altered expression of ZIP12 was linked to congenital malformations caused by zinc deficiency [280]. Since T cell maturation, proliferation, differentiation, and function are highly zinc-dependent, and zinc deficiency impairs Th cell function [45,71,72,283,284], schizophrenia might be triggered due to malfunction of Th differentiation and function.

8.4. Zinc and Depression

Depression is a psychiatric disorder associated with high morbidity and mortality, being the cause of about 50–70% of all suicides [285]. The World Health Organization has predicted that depression will be the second leading contributor to the global burden of disease by 2020 [286]. Common symptoms for depression are sadness, exhaustion, and lack of interest in the daily activities of living [287].

In patients suffering from depression, increased pro-inflammatory cytokine concentrations, such as IL-1β, IL-6, and IFNγ, were found [288]. Furthermore, serum hypozincemia was observed, which was normalized by effective anti-depressant treatment. Interestingly, high amounts of zinc are present in the brain, particularly in the hippocampus and cerebral cortex [26,289]. It was shown that zinc deficiency negatively influenced brain zinc homeostasis and resulted in impaired mental function, altered behavior, learning, and susceptibility to epileptic convulsions [287,290]. Interestingly, zinc administration exhibited antidepressant-like effects when tested for antidepressant activity. Zinc administration produced antidepressant-like effects in the "forced swim test", in both mice and rats, and in the tail suspension test [287]. During anti-depressant therapy in humans, Treg cells were induced while IL-1β level decreased concurrently [291]. Zinc administration induced Treg cells [72,170], leading to a dampened pro-inflammatory immune reaction. Thus, zinc deficiency can favor the development of depression, whereas zinc administration can exhibit antidepressant-like activity.

8.5. Zinc and Multiple Sclerosis

Worldwide there are about two million patients diagnosed with Multiple Sclerosis (MS), making it the most common chronic-inflammatory disease of the central nervous system (CNS) [22]. MS is characterized by: (1) inflammation of the CNS; (2) destruction of the myelin sheath, which surrounds the neuronal axon and forms an electrically insulating layer that is essential for proper signal transmission and thus for the function of the nervous system; and (3) paralysis, mediated by myelin-specific $CD4^+$ Th cells.

One possible hypothesis for MS progression is believed to be due to T cell infiltration into the CNS boosting inflammation. Interestingly, T cell function is strictly regulated by zinc concentration, and zinc deficiency caused malfunctions and altered T cell differentiation, leading to an imbalanced immune response. Indeed, failures in zinc homeostasis were associated with the development of MS. In this regard, dietary zinc deficiency, as well as increased zinc uptake, were shown to be link with increased risk of MS and the development of pathogenic pro-inflammatory T cells in experimental autoimmune encephalomyelitis (EAE) [20,292,293]. Patients suffering from MS displayed lower plasma zinc levels and lower zinc concentrations in chronic MS lesions [21,22,294]. Zinc administration in EAE

FOI 26-3132 - Document 5

diminished EAE symptoms by inducing Treg cells and diminishing Th17 cells. Thus, the pro-tolerogenic immunoreaction was favored while the pro-inflammatory was dampened. In line with that, zinc signals are well known to be of central importance in keeping unwanted T cell-mediated immunoreactions in check, by suppressing apoptosis, immune cell proliferation, and pro-inflammatory cytokine production as well as IL-2, IL-10 and IL-17 production [214,295–298]. Therefore, adding zinc supplementation to current therapeutic standard treatments can be considered as potentially effective for patients suffering from MS.

8.6. Zinc and Ehlers-Danlos Syndrome

Ehlers-Danlos syndrome (EDS) is a human disorder characterized by joint hypermobility, hyperelasticity of the skin, progressive kyphoscoliosis, and severe hypotonia of skeletal muscles [299]. The zinc transporter ZIP13 plays crucial roles in bone, tooth, and connective tissue development. Disturbed zinc homeostasis due to ZIP13 malfunction appeared to contribute to EDS pathogenesis, whereby the mutant ZIP13 protein was quickly degraded [300,301].

In addition to the identification of the EDS/ZIP13 relationship in humans, ZIP13 knockout mice were investigated. Those showed an abnormal systemic growth, bone growth, abnormal cartilage development, reduced dentin and alveolar bone, abnormal craniofacial and decreased dermal and corneal stromal collagen. At the molecular level, the absence of ZIP13 provoked a dysregulation of bone morphogenetic protein (BMP)-mediated gene expression [302]. Since BMP signaling covers a high variety of target genes, bone, tooth, and craniofacial development was impaired. Both BMP and transforming growth factor (TGF)-β signal via the TGF-β receptor and activate Smad signaling molecules containing a zinc-binding motif in the DNA binding domain, thus making Smad signaling a potential target of zinc signaling [303]. Therefore, ZIP13 might influence zinc availability for that motif.

Another zinc-conditioned target in BMP signaling is Smad protein phosphorylation. Smad proteins need to be phosphorylated for translocation to the nucleus. Altered intracellular free zinc concentrations led to inhibition of phosphatase activity, subsequently increasing Smad phosphorylation, as demonstrated recently [168]. Smad signaling is highly important for adequate development of Treg and for tolerance induction. If this mechanism is not working accurately, inflammation is triggered. Elevated levels of inflammatory markers, ROS and MMPs are well known to cause tissue injury in numerous tissues. Since EDS is accompanied by disturbed collagen synthesis, and MMPs are one key factor in collagenolysis, MMP malfunction can be considered as a possible explanation for EDS. In this context, zinc signals are crucial because inflammation, as well as MMP hyperfunction, accompanies serum hypozincemia [5,63,66].

Hence, altered intracellular zinc level in EDS might be due to ZIP13 malfunction. For this reason, a possible therapeutic approach could be based on the regulation of the mutant ZIP13 protein stability [300].

8.7. Zinc in Inflammatory Disease and Sepsis

ZIP8 influences the innate as well as adaptive immune function during bacterial infection and inflammation and a multitude of other diseases are at least related to ZIP8 malfunction [304,305]. In lung epithelia, ZIP8 is crucial for cyto-protection upon inflammation, mediated by zinc signals [306,307]. Moreover, ZIP8 controls the expression of the pro-inflammatory cytokine IFNγ and its immunological function through the regulation of zinc release from lysosomes [101]. Besides adaptive immunity, ZIP8 also regulates innate immunity through zinc-mediated inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) function in macrophages and monocytes [202].

Besides local inflammation, as described above, systemic inflammation, as observed in sepsis, is frequently observed in the population. In this regard, altered zinc transport mechanisms seem to contribute to the inflammatory effects of sepsis. Most likely, ZIP14, which is inducible by pro-inflammatory stimuli, influences zinc metabolism during sepsis and serves as a target for therapy [63]. Zinc signals act in an anti-inflammatory manner during sepsis by attenuating the

FOI 26-3132 - Document 5

pro-inflammatory response, due to cellular zinc uptake by ZIP14. Hence, zinc signals and proper ZIP14 function are essential in pro-inflammatory responses, and zinc deficiency is strongly associated with an elevated risk for exaggerated inflammation and mortality due to sepsis [63,65].

8.8. Zinc in Lactation

Some breast-fed full-term infants show apparent clinical showing symptoms similar to Acrodermatitis Enteropathica, such as growth retardation, dermatitis, diarrhea, and alopecia. Studies showed this was the result of zinc deficiency caused by low zinc concentrations in the breast milk [308–310]. Zinc in breast milk is essential for the growth and health of neonates, since infantile zinc deficiency is associated with autism spectrum disorders [247]. Genetic analyses indicated that malfunction of ZnT2 was responsible for this condition [309,311,312]. Symptoms can be alleviated by zinc supplementation to the infant, but not to the mother. Interestingly, similar observations were described for mice bearing malfunctioning ZnT4 [313], whereby decreased zinc content in milk was detectable [314]. With time, low zinc-concentrations were lethal for suckling pups. ZnT4 has profound consequences on mammary epithelial cell function and negatively influences nutrition secretion into the milk, and additionally promotes tissue remodeling in the mammary gland during early lactation [315]. Animal studies in mice bearing a malfunction of ZnT4, showed these defects to be associated with decreased Akt expression, and Signal transducer and activator of transcription 5 (STAT5) activation. Moreover, in humans a significantly decreased ZnT5 and ZnT6 mRNA expression was found in fibroblasts and lymphoblasts of mothers secreting zinc-deficient milk [316]. Thus, other ZnT proteins are probably involved in the etiology of this disease. Therefore, future studies are needed to elucidate the entire molecular mechanism of the regulation of ZnT2 expression in mammary epithelial cells to protect breast-fed infants against zinc deficiency and to guarantee proper growth and health of neonates.

8.9. Zinc and Diabetes

Diabetes is a chronic disease which damages blood vessels, heart, eyes, kidneys, and nerves. About 340 million people are affected worldwide making diabetes a major cause of morbidity and mortality [317]. Up to ninety percent of patients suffering of diabetes are diagnosed as having type 2 diabetes mellitus (T2DM) and 10–15% type 1 diabetes (T1DM). Whereas T2DM is mainly related on lifestyle, T1DM is an autoimmune disease with an early onset [318]. DM is characterized by loss of glycemic control, disturbed metabolic homeostasis through deterioration of pancreatic β-cell function, and insulin resistance. Besides behavioral factors, hereditary factors also play an important role in determining T2DM risk. The latter include a malfunctioning ZnT8 gene [105]. Thus, ZnT8 transporter is associated with both T1DM and T2DM, respectively [319,320]. Mice that are deficient in ZnT8 have impaired insulin secretion, impaired crystal formation and clear insulin rapidly from the liver [321,322]. Regarding T1DM, ZnT8 autoantibodies are present in 60–80% of patients at the onset of clinical disease [319,323,324]. However, ZnT8 was the last autoantigen uncovered in T1DM, whereas insulin, 65kDa glutamic acid decarboxylase (GAD65) and insulinoma-associated protein 2 (IA-2) where already established long before [318]. Since the prevalence of autoantibodies against ZnT8 has a similar frequency to standard T1DM autoantigens, ZnT8 can be used additionally to detect diabetes-related autoimmunity [325,326].

In diabetes, zinc plays a critical role in the formation of insulin crystals in β-cells. Moreover, both insulin and zinc released from β-cells are considered to have crucial paracrine and/or autocrine effects, leading to modulation of various molecular mechanisms [327,328]. In these processes, ZnT8 is essential to guarantee the transport of zinc into secretory vesicles that contain insulin. Those are referred to as insulin granules. The importance of ZnT8 was highlighted by studies investigating ZnT8 knockout mice [322,329,330]. These had significantly decreased zinc contents in β-cells and show only fragmented immature zinc/insulin crystals. Additionally, studies using β-cell-specific, as well as

FOI 26-3132 - Document 5

α-cell-specific ZnT8, knockout mice revealed that ZnT8 deficiency contributed to a higher incidence of T2DM through β-cell- and non-β-cell-specific effects, respectively [330,331].

Dietary zinc supplementation in mice in vivo was able to attenuate hyperglycemia and hyperinsulinemia, and induced elevated pancreatic zinc concentrations [117], and improved glucose clearance in diabetic rats and mice was observed [123,146]. In line with that, it is well known that zinc-deficient animals are less sensitive to insulin [147]. Oral zinc administration in both mice and humans improves glycemic control in T1DM and T2DM patients, respectively [3]. Since plasma zinc levels in T1DM and T2DM patients were reduced compared to healthy individuals, oral zinc supplementation might be useful as an adjunct therapy to promoting insulin signaling [332,333]. Autoantibody production observed in T1DM might be dampened by zinc supplementation, since especially T cell-dependent antibody production is affected by zinc deficiency [209]. Furthermore, zinc intake may stabilize regulatory T cells as discussed above [72]. This is important in relation to environmental factors such as zinc in drinking water and soils. Although T1DM is clearly related to genetic factors such as HLA-DR3-DQ2 and HLA-DR4-DQ8 [318], the risk of onset of T1DM is associated with low levels of zinc in drinking water and soils [233,334]. Moreover, zinc deficiency triggers the IL-6-induced phosphorylation of STAT3 [145], thereby pathologically enhancing B cell differentiation into antibody-producing plasma cells and autoantibody production. This is due to IL-6 overproduction that is associated with autoantibody production [335].

However, further molecular research needs to be done in this field. ZnT8 may be engaged in the pathogenesis of T2DM in multiple ways and further molecular research is needed to reveal the complete molecular mechanisms responsible for the increased risk to develop DM.

8.10. Zinc and Alzheimer's Disease

Alzheimer's disease (AD) was first described in 1906 and accounts nowadays for 60–80% of all dementia cases reported [336]. AD is a chronic neurodegenerative disorder representing the most prevalent form of dementia in modern society. Patients suffering of AD show a progressive cognitive decline. Post-mortem examinations uncovered extracellular deposits, referred as plaques, intracellular deposits, referred as neurofibrillary tangles (NFTs), as well as a significant thinning of cortical grey matter, enlargement of the ventricular spaces, and an overall atrophy of the brain [5].

It is well known that a zinc dyshomeostasis is present in AD that might trigger AD-related pathology and symptoms. While the ultimate cause of this zinc imbalance remains to be fully elucidated yet, a dysregulation in various metal storage and transport proteins is likely [337].

An imbalanced zinc homeostasis in the hippocampal/parahippocampal gyrus brain area is related to preclinical stage of AD. This is probable due to an increased expression of the zinc transport proteins ZnT4, and ZnT6, and decreased expression of ZnT10 altering zinc concentration and zinc signals in the hippocampal/parahippocampal gyrus [105,338,339]. In addition to that, ZnT1 expression was observed to be reduced in the same brain region individual's mild cognitive impairment. However, ZnT1 expression was increased in early and late stage AD compared to healthy controls [340]. In accordance to that, zinc-associated activation of tyrosine kinases has been uncovered in patients suffering from AD, leading to misdirection of signaling cascades in neuronal cells of affected patients [112]. Moreover, zinc buffering by MT or calcineurin seems to be altered in AD. Some reports claim changes in MT expression, especially in MT-III [341,342]. However, this is still controversy discussed [343]. Additionally, more recent studies uncovered calcineurin activation to be linked to beta-amyloid (Aβ)-mediated neurodegeneration in AD [344,345]. Analysis of brain tissue uncovered a significant decrease in zinc levels found in AD patients' neocortex, hippocampus, medial temporal gyrus and thalamus, and superior frontal and parietal gyri [346]. However, these findings are still under discussion [347,348]. Interestingly, high zinc concentrations seem to trigger AD progression, however low zinc concentrations at ratios of 1:0.1 (Aβ/Zn ratio) and 1:0.01 have a protective effect against Aβ toxicity [349,350]. This is in line with findings of Lee et al., showing that ZnT3 KO mice having less vesicular zinc, simultaneously have less cortical and hippocampal plaques [351].

FOI 26-3132 - Document 5

Another explanation of the zinc-driven protective effect against Aβ toxicity is due to zinc binding to Aβ peptide instead of copper. When copper is bound to the Aβ peptide ROS production and cellular damage is triggered [352]. Hence, cellular damage can be reduced by zinc binding to the Aβ peptide.

In summary, all studies uncover that a disturbed zinc homeostasis may play a role in the pathogenesis of AD. Nevertheless, more research is necessary to uncover potential therapeutic targets to improve patients' lives.

9. Conclusions

Zinc flux, zinc wave, and homeostatic zinc signals control the adequate function of innate as well as adaptive immunity. On the one hand, zinc deficiency causes severe impairment of immune function, comprising the adaptive as well as the innate immune system. On the other hand, high zinc excess provokes an impairment of the immune system comparable to zinc deficiency (see Figure 5). This is why a balanced zinc homeostasis is crucial for either defending against invading pathogens or protecting the human body against an overreactive immune system causing autoimmune diseases, chronic inflammation or allergies. In this regard, zinc can be considered as a gatekeeper of the immune system, since the adequate function of virtually all immune cells is highly zinc-dependent. Knowledge about the molecular mechanisms in zinc-regulated immune reactions is growing and inherited disorders such as Acrodermatitis Enteropathica, as well as nutrition-related immunological malfunctions observed in the elderly, can be treated by appropriate zinc administration. Thus, zinc can be seen as potential therapeutic for clinical use to influence beneficially the well-being of patient's suffering from immune diseases. However, to completely understand the complex-regulated zinc-triggered immunoreactions, more research will be necessary.

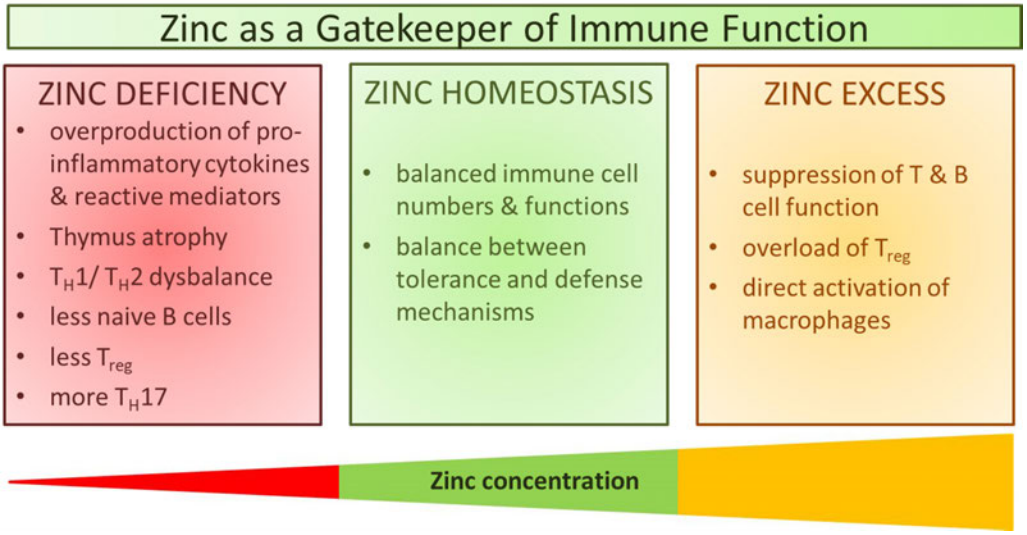


Figure 5. Influence of zinc status on the overall immune function. Adequate zinc homeostasis is essential for a well-functioning immune system. Zinc deficiency as well as zinc excess lead to malfunction of the adaptive and innate immune system, eventually resulting in the development of numerous immune diseases.

Acknowledgments: We thank Alan Slusarenko, RWTH Aachen for language editing. Lothar Rink is a member of Zinc Net.

Author Contributions: I.W. wrote Section 5, Section 6, and Section 8, and designed Figures 2–4. M.M. wrote Sections 1–4, 7 and 9, and designed Figure 1. L.R. made substantial contributions to the conception and design of the manuscript and substantively revised it.

Conflicts of Interest: The authors declare that they have no conflict of interest.

FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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frontiers in Immunology

FOI 26-3132 - Document 5
PERSPECTIVE
published: 10 July 2020
doi: 10.3389/fimmu.2020.01712

The Potential Impact of Zinc Supplementation on COVID-19 Pathogenesis

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During the current corona pandemic, new therapeutic options against this viral disease are urgently desired. Due to the rapid spread and immense number of affected individuals worldwide, cost-effective, globally available, and safe options with minimal side effects and simple application are extremely warranted. This review will therefore discuss the potential of zinc as preventive and therapeutic agent alone or in combination with other strategies, as zinc meets all the above described criteria. While a variety of data on the association of the individual zinc status with viral and respiratory tract infections are available, study evidence regarding COVID-19 is so far missing but can be assumed as was indicated by others and is detailed in this perspective, focusing on re-balancing of the immune response by zinc supplementation. Especially, the role of zinc in viral-induced vascular complications has barely been discussed, so far. Interestingly, most of the risk groups described for COVID-19 are at the same time groups that were associated with zinc deficiency. As zinc is essential to preserve natural tissue barriers such as the respiratory epithelium, preventing pathogen entry, for a balanced function of the immune system and the redox system, zinc deficiency can probably be added to the factors predisposing individuals to infection and detrimental progression of COVID-19. Finally, due to its direct antiviral properties, it can be assumed that zinc administration is beneficial for most of the population, especially those with suboptimal zinc status.

Keywords: zinc, COVID-19, SARS-CoV2, 2019-nCoV, coronaviridae, zinc deficiency, impaired immune system

OPEN ACCESS

Edited by:
Francisco José Pérez-Cano, University of Barcelona, Spain

Reviewed by:
Anastasia N. Vlasova, The Ohio State University, United States
Alberto Finamore, Council for Agricultural and Economics Research (CREA), Italy

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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *18 May 2020*
Accepted: *26 June 2020*
Published: *10 July 2020*

Citation:
Wessels I, Rolles B and Rink L (2020) The Potential Impact of Zinc Supplementation on COVID-19 Pathogenesis. Front. Immunol. 11:1712. doi: 10.3389/fimmu.2020.01712

INTRODUCTION

The importance of the trace element zinc for the development and function of the immune system across all kinds of species has been proven in numerous studies (1–3). As zinc deficiency results in altered numbers and dysfunction of all immune cells, subjects with suboptimal zinc state have an increased risk for infectious diseases, autoimmune disorders, and cancer (3–6). In addition to malnutrition, risk groups for zinc deficiency include the elderly and patients with various inflammatory and autoimmune diseases, which will be discussed in detail later in the article (7, 8). As mild zinc deficiency is largely sub-clinical, it is unnoticed in most people. However, the World Health Organization (WHO) assumes that at least one third of the world population is affected by zinc deficiency (9). The fact that zinc deficiency is responsible for 16% of all deep respiratory infections world-wide (9) provides a first strong hint on a link of zinc deficiency with the risk of infection and severe progression of COVID-19 and suggests potential benefits of zinc supplementation.

The most common symptoms of COVID-19 are impaired smell and taste, fever, cough, sore throat, general weakness, pain as aching limbs, runny nose, and in some cases diarrhea (10). In the subsequent chapters, we will associate most of those symptoms with altered zinc homeostasis and explain how zinc might prevent or attenuate those symptoms, as summarized in **Figure 1**, and thus should be regarded as promising cost-effective, globally available therapeutic approach for COVID-19 patients, for which minimal to no side effects are known.

ZINC PROTECTS THE HUMAN BODY FROM ENTERING OF THE VIRUS

The entry of infectious agents into the human body is prevented by tissue barriers equipped with cilia and mucus, anti-microbial peptides like lysozymes and interferons. Regarding SARS-CoV2, the angiotensin-converting enzyme 2 (ACE2) and the cellular protease TMPRSS2 are the major mechanism for entering the cells (11).

a) Mucociliar clearance of viruses is affected by zinc
   Infections with coronaviruses go along with damage of the ciliated epithelium and ciliary dyskinesia consecutively impairing the mucociliar clearance (12). It was shown that physiological concentrations of zinc increase ciliary beat frequency (13). Moreover, zinc supplementation in zinc deficient rats had a positive effect on the number and the length of bronchial cilia (14) (**Figure 1.4**). Improved ciliary clearance does not only improve the removal of virus particle, it also reduces the risk of secondary bacterial infections, as discussed later. Alterations of the extracellular matrix, as monitored by increased epidermal growth factor and proliferating cell nuclear antigen (PCNA) immunostaining of rat lungs during zinc deficiency have also been described (15).
b) Zinc is essential for preserving tissue barriers
   Disturbances in the integrity of the respiratory epithelia facilitate the entry of the virus as well as co-infecting pathogens and can lead to pathogens entering the blood stream. An *ex-vivo* model of the chronic obstructive pulmonary disease (COPD) showed that decreasing zinc levels raised the leakage of the epithelium of the respiratory tract (16), while zinc supplementation improved lung integrity in a murine model of acute lung injury *in vivo* (17). Increased apoptosis and E-cadherin/beta-catenin proteolysis were amongst the underlying mechanisms (17–19). The expression of tight junction proteins like Claudin-1 and ZO-1 was found to be zinc-dependent, offering another explanation for zinc's positive effects on lung integrity (16). In addition, an inhibitory effect of zinc on LFA-1/ICAM-1 interaction weakened inflammation in the respiratory tract via reduction of leukocyte recruitment (20). Furthermore, high zinc levels improved the tolerance of the lung towards damage induced by mechanical ventilation (21) (**Figure 1.4**).
c) Zinc-dependent alterations in gene expression by pneumocytes could affect viral entering
   ACE-2, mainly expressed on pneumocytes type 2, is a zinc-metalloenzyme. Zinc binds to its active center and is thus essential for its enzymatic activity. If zinc binding also affects the molecular structure of ACE-2 and thereby its binding affinity to the virus, remains to be tested (22, 23). However, this is likely as zinc is important for stabilizing protein structures and altering substrate affinity of various metalloproteins (24, 25). Finally, zinc homeostasis might affect ACE-2 expression, as zinc-dependent expression was reported for other zinc-metalloenzyme such as metallothionein and matrix metalloproteinases (26). This suggestion is strengthened by the finding that ACE-2 expression is regulated by Sirt-1 (27, 28). As zinc decreases Sirt-1 activity (27), it might decrease ACE-2 expression and thus viral entry into the cell (**Figure 1.2**).

A lack of adequate secretion of type I and type II interferons was reported for COVID-19 patients (29). For human interferon alpha (IFN-α) it was shown that zinc supplementation can reconstitute its expression by leukocytes and potentiates its anti-viral effect via JAK/STAT1 signaling as observed for rhinovirus-infected cells (30, 31). However, as it was suggested that SARS-CoV2 might take advantage of the interferon-dependent expression of ACE2, which was recently addressed by Ziegler et al. (32), the overall effects of zinc need to be carefully evaluated in future studies.

ZINC DIRECTLY INHIBITS VIRAL REPLICATION

As a virus, SARS-CoV2 is highly dependent on the metabolism of the host cell. Direct antiviral effects of zinc have been demonstrated in various cases, which was reviewed in great detail (33–37). Examples include coronaviridae, picornavirus, papilloma virus, metapneumovirus, rhinovirus, herpes simplex virus, varicella-zoster virus, respiratory syncytial virus, human immunodeficiency virus (HIV), and the hepatitis C virus (34, 35, 37–39). It was suggested that zinc can prevent fusion with the host membrane, decreases the viral polymerase function, impairs protein translation and processing, blocks viral particle release, and destabilizes the viral envelope (35, 37, 40). Low-dose zinc supplementation together with small concentrations of the zinc ionophores pyrithione or hinokitol decreased RNA synthesis in influenza, poliovirus, picornavirus, the equine arteritis virus, and SARS-CoV by directly inhibiting the RNA-dependent RNA polymerase of the virus (34, 41). There is evidence that zinc can enhance the effect of chloroquine, another known zinc ionophore, while zinc ionophores like epigallocatechin-gallate or quercetin remain to be tested (42–45). There are close similarities of SARS-CoV2 and other coronaviridae like SARS-CoV and Middle East respiratory syndrome-related coronavirus (MERS-CoV) (46). Also, the alcohol-aversive drug disulfiram can bind the papain-like proteases of SARS-CoV and MERS-CoV resulting in release of cysteine-bound zinc that results in protein destabilization (47). Detailed studies on zinc's effect specifically on SARS-CoV2 are highly required (**Figure 1.3**).

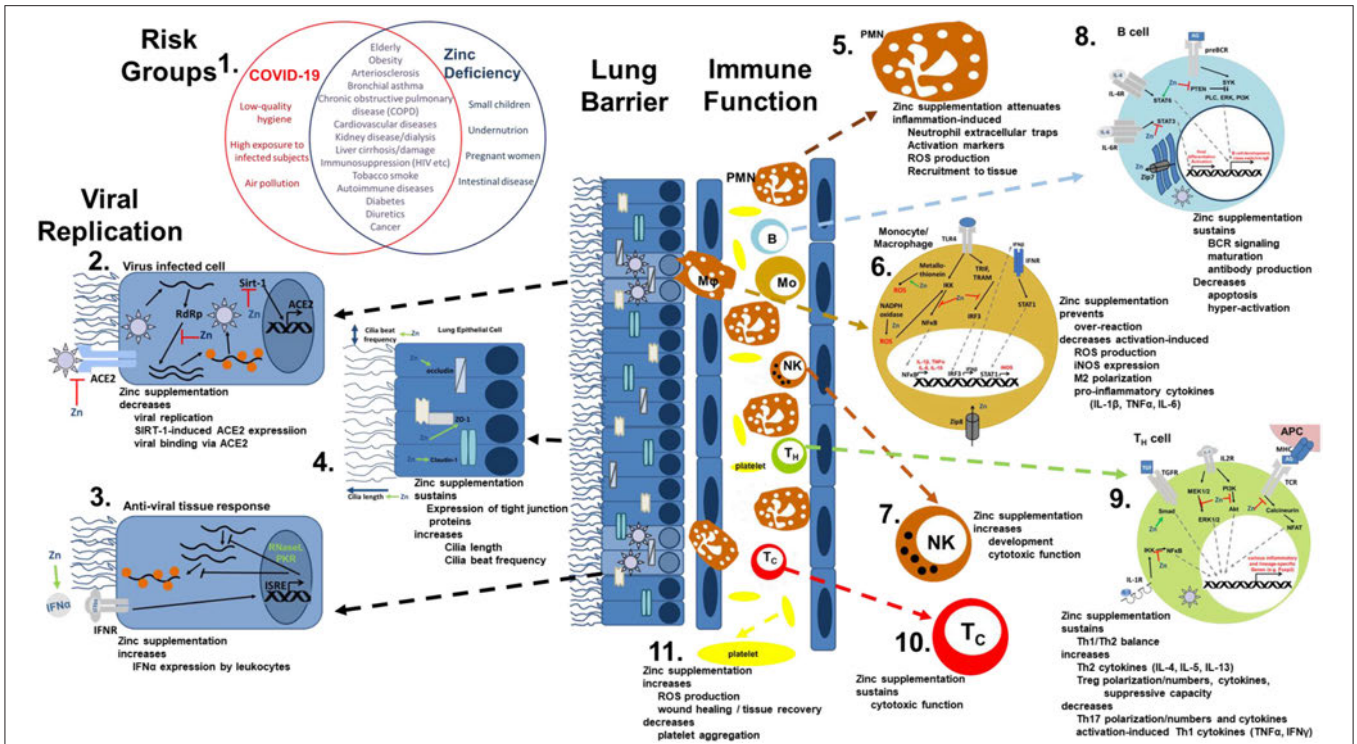


FIGURE 1 | Viral mechanism of COVID-19 and how they might be opposed by zinc data. **(1)** There is an impressive intersection of known risk factors for zinc deficiency (blue circle) and the predisposition for a severe COVID-19 infection (red circle). **(2,3)** Zinc (Zn) supplementation might already prevent the viral entry and also suppresses its replication, while it supports the anti-viral response of the host cells. **(4)** As zinc is known to increase ciliary length and movements and also sustains tissue integrity, entrance of the virus is impeded. **(5–10)** The importance of zinc on the development and function of the immune cells is manifold. It should be underlined, that zinc's effects should not generally be described as activating or inhibiting, as zinc in various cases normalizes overshooting immune reactions and balances the ratios of the various immune cell types. Zinc thus prevents for example that high levels of inflammatory mediators including reactive oxygen and nitrogen species destroy the host tissue. **(11)** On first view it appears contradicting, that zinc increases activation induced production of reactive oxygen species in platelets, while it is generally considered as anti-oxidative. However, in case of platelets, up to a certain threshold, ROS production is essential, as it can prevent the formation of platelet aggregates. In summary, zinc therefore might be able to prevent vascular complications observed in COVID-19 patients. Details for each point can be found in the text. ACE2, angiotensin converting enzyme 2; AG, antigen; IFN, interferon; IFNR, interferon receptor; ISRE, interferon-sensitive response element; APC, antigen presenting cell; IKK, IκB kinase; IL, interleukin; iNOS, inducible nitric oxide synthase; IRF3, IFN regulatory factor 3; MHC, major histocompatibility complex; MEK1/2, mitogen-activated protein kinase kinase 1/2; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor kappa B; PKR, protein kinase R; Akt, protein kinase B; PI3K, phosphatidylinositol-3 kinases; ROS, reactive oxygen species; RdRP, RNA-dependent RNA polymerase; RNase L, ribonuclease L; Sirt-, Sirtuin 1; STAT, signal transducer and activators of transcription; TCR, T cell receptor; Tc, cytotoxic T cell; TH, helper T cell; TGF, transforming growth factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β; TLR, toll-like receptor; TNF, tumor necrosis factor; Zip, Zrt- and Irt-like protein; ZO-1, zona occludens.

ZINC BALANCES THE IMMUNE RESPONSE DURING INFECTIOUS DISEASES

One of the hallmarks of COVID-19 is an imbalanced immune response (48). Due to hyper-inflammation, immune products including pro-inflammatory cytokines like interleukin (IL)-6, C-reactive protein (CRP), tumor necrosis factor (TNF)α and IL-1β (summarized as cytokine storm or cytokine release syndrome), reactive oxygen, and nitrogen species in connection with the recruitment of high numbers of strongly activated immune cells to the lungs, the destruction of the tissue, permanent lung damage and death due to systemic inflammation, and organ failure are expected, while the anti-inflammatory response is insufficient (48–52). A high number of patients develop an acute respiratory distress syndrome (ARDS) accompanied by high alveolar leakage leading to alveolar and interstitial edema with severely limited oxygen exchange (53). Advanced SARS-CoV2 infections are characterized by a systemic involvement with organ complications and accompanying cytokine storm (52, 54).

There is no doubt on the anti-inflammatory and anti-oxidative properties of zinc and underlying mechanisms have been the focus of numerous studies (1–3, 6, 55–60). A detailed description of zinc metabolism in airway epithelium and during inflammation of the airways has been published by Zalewski et al. (61). On the other hand, zinc deficiency was associated with elevated levels of pro-inflammatory mediators, increased reactive oxygen species (ROS) levels and pre-disposing for severe progression of inflammatory diseases, especially those affecting the lung, often reversible by zinc supplementation (6, 17, 56, 62–66) (**Figures 1.5,1.6**). As one example, exposure to organic dust increased lung damage, inflammation and

FOI 26-3132 - Document 5

macrophage hyper-activation in animals with zinc deficiency, predisposing these animals to pulmonary fibrosis, while zinc supplementation 24 h before induction of acute lung injury significantly attenuated the inflammatory reaction and tissue damage (17, 67). Regarding systemic inflammatory diseases the number of studies showing benefits of especially preventive zinc supplementation is constantly increasing (17, 18, 58, 65, 68). Amongst the underlying mechanism, zinc's role as second messenger and importance in regulating intracellular signaling as detailed in **Figure 1** were described as well as zinc's effects on the epigenome (56, 57, 69–74).

Furthermore, leukocytosis with neutrophilia and lymphopenia, especially affecting $CD8^+$ T cells, were associated with poor prognosis of COVID-19 and the recovery of lymphocyte counts lead to clinical recovery (75, 76). Similar changes in lymphopoiesis and myelopoiesis have been described in zinc deficient rodents, which were normalized when zinc was supplemented (17, 19). Circulating and lung-resident T cells from COVID-19 patients showed increased expression of markers for T cell exhaustion like Tim-3 and PD-1 (77). The extent of these changes had an impact on the patient's prognosis (50). During the past decades, an immense literature was generated on the need of zinc for lymphocyte development and function and that zinc supplementation (6, 19, 63, 64, 78, 79) can reverse lymphopenia. Enumerating all findings and underlying mechanisms is beyond the scope of this article, and a lot of aspects have been discussed in related publications (36) but as one of the many key roles of zinc in the context of T cell function, zinc is indispensable in the signal cascade of the T cell receptor and IL-2 as a second messenger (78, 80) (**Figure 1.9**). The B cell compartment also strongly benefits from a balanced zinc homeostasis, as zinc is required for B cell maturation and function (72, 81) (**Figure 1.8**). Also important to mention, but neglected by previous related articles, is that there is evidence (82, 83) that SARS-CoV2 can directly infect T cells as well as B cells and impair their cell specific function. This could explain the impact of SARS-CoV2 infection on lymphoid tissues like the human spleen and lymph nodes (84). However, as data are limited to *in vitro* experiments, this needs to be verified *in vivo* as well as if zinc affects the virus-induced changes in T and B cells.

Additionally, granulocytes play a vital role during the inflammation-induced destruction of the lung (85). Recent evidence suggests that lipopolysaccharide-induced hyper-activation, recruitment and formation of neutrophil extracellular traps are attenuated by zinc supplementation *in vivo* and that cytokine expression, phagocytosis and burst, chemotaxis and degranulation, and intracellular signaling are zinc regulated (17, 86, 87) (**Figure 1.5**). Important defense mechanisms of the innate immunity include the toll like receptors. For instance, *in silico* data suggest that toll-like receptor (TLR)-4 can potentially recognize outer components of SARS-CoV2's like the viral spikes (88), while intracellular receptors including TLR3, TLR7/8, and TLR9 can recognize viral dsRNA, ssRNA, and unmethylated CpG DNA respectively (89–92). Intranasal pretreatment with a TLR3 agonist and, to a lesser extent, with TLR9, TLR7/8, or TLR4 agonists, provided a high level of protection against infections by SARS coronavirus and influence virus in mice, suggesting that TLR signaling can induce protective antiviral immunity (93). This might be a completely novel approach to consider regarding COVID-19 as well. Zinc is an essential regulator in TLR-4- and TLR-3-induced signaling in innate immune cells (94). Thus, zinc deficiency potentially disturbs the innate immune response toward SARS-CoV2, enabling the virus to easily spread throughout the host without an adequate immune response (**Figure 1.6**).

Clinical improvement of COVID-19 patients was correlated to an increase of $CD14^+$ monocytes and NK cells in the recovery phase (48). For a physiological inflammatory response and phagocytic activity macrophages need sufficient intracellular zinc levels (1). In addition, for NK cells and cytotoxic T cells it was shown that zinc supplementation increased their cytotoxicity toward target cells (1, 2, 95) (**Figures 1.7,1.10**).

In summary zinc's (re-)balancing power regarding immune cell numbers and functions might be highly beneficial in regard to therapy of COVID-19.

ZINC SUPPLEMENTATION IN RESPIRATORY INFECTIONS

Our suggested benefits of zinc supplementation to prevent and treat COVID-19 are supported by a row of successful supplementation studies focusing on respiratory tract infection, of which we listed some selected publications in **Table 1**. In most cases, prophylactic zinc supplementation was more effective than therapeutic proceedings (106–108, 111). Up to 30% of the everyday respiratory infections, briefly named "common cold," are due to infections with coronaviruses (112). Studies showed reduced symptom severity, reduced frequency, and duration of the common cold after zinc administration (99, 100, 113, 114) depending on dosage, zinc compound and the start time after initial symptoms (115). Most importantly, zinc supplementation of children revealed significant benefits in various studies (96, 106) and reduced 15% pneumonia-specific morality and 19% of pneumonia morbidity in developing countries (116).

RISK GROUPS AND SYMPTOMS OF COVID-19 AND ZINC DEFICIENCY REVEAL A LARGE OVERLAP

As illustrated in **Figure 1.1**, the intersection between risk groups of COVID-19 and zinc deficiency is impressive. In patients with chronic obstructive pulmonary disease (COPD), bronchial asthma, cardiovascular diseases, autoimmune diseases, kidney diseases, dialysis, obesity, diabetes, cancer, atherosclerosis, liver cirrhosis, immunosuppression, and known liver damage low serum zinc levels are regularly observed (4, 117). At the same time, these groups are particular at risk for COVID-19 (10, 51, 118, 119). For example 57.5% elderly and nursing home residents in the U.S., for which high incidence of respiratory tract infections is described, showed significantly decreased zinc intake levels and are considered subjects with high risk regarding COVID-19 (120). Moreover, other studies showed that serum zinc levels were inversely correlated with pneumonia and cystic

FOI 26-3132 - Document 5

TABLE 1 | Selected zinc supplementation studies in respiratory infections.

Compound	Conc. [mg/d]	Duration	Disease	Effect	References
Treatment					
Zinc bis-glycinate	30 (elemental)	Max 7 days/dis-charge from the hospital	Lower RTI (Children)	Reduction of days of ALRI and shorter hospital stay	(96)
Zinc acetate	20	5d	Lower RTI (children)	Increased recovery rates (boys)	(97)
Zinc gluconate	10	6 mo	Lower RTI (children)	Decreased episodes of infection, more infection free days	(98)
Zinc gluconate Zinc actetate Gluconate nasal gel SULFITE nasal spray	60–313 76.8–102.4 2.1 0.044	Until symptoms are gone	Common cold	Variable results but generally reduced duration if supplementation started within first 24 h	Meta-study of 16 studies (99)
Zinc acetate vs. zinc gluconate	80–92 192–207	Until symptoms are gone	Common cold	<75 mg/day: reduced duration; zinc acetate better than gluconate	Meta-study of 7 studies (100)
Zinc gluconate	30 (elemental)	12 mo	Cystic fibrosis (children)	Reduced duration of antibiotics	(101)
Prophylactic					
Zinc sulfate	20 (elemental)	2 wk/6 mo follow-up	Lower RTI (children)	Reduced morbidity	(102)
Zinc sulfate	20 to ZD children	14d, 6 mo follow-up	Upper and lower RTI (children)	Decreased incidence and duration of upper and lower RTI	(103)
Zinc oxide	5	12 mo	Upper RTI (children)	Decreased incidence	(104)
Zinc gluconate	10	6 mo	Lower RTI	Decreased incidence	(105)
Zinc acetate, gluconate, methionine, sulfate	Min 70 mg/wk	>3 mo	Lower RTI	Decreased incidence (depending on criteria)	Meta-study of 10 studies (106)
Zinc in mineral mix	6 (f)−7.5 (m)	12 mo	Naturally occurring pneumonia	Decreased incidence and duration, decreased duration of antimicrobial therapy	(107)
Zinc sulfate	60–90	12 mo	Ventilation associated pneumonia	Decreased incidence	(108)
Zinc gluconate	Up to 12x 23 mg/d	Until symptoms are gone	Common cold	Decreased clinical score	(109)
Zinc sulfate	15	7 mo	Common cold	Decreased incidence	(110)
Murine models					
Zinc-enriched rodent diet	ZD: 50 ppm–ZS: 100 ppm	18d ZD followed by 3d ZS	Sepsis-induced ALI	Decrease in inflammation, lung damage, and mortality (vs. ZD mice)	(68)
Zinc aspartate	30 μg/ mouse	24 h	Acute lung injury (LPS inhalation), mice	Decreased hyper-inflammation, tissue damage	(17)

Conc, concentration; d, day; mo, months; ref, reference; RTI, respiratory tract infection; ZD, zinc deficiency; ZS, zinc supplementation; wk, week.
Single studies are not included in the meta-studies.

fibrosis (121, 122). On the other hand, zinc supplementation was able to reconstitute immune function in elderly and zinc deficient individuals (107, 123), which remains to be addressed for SARS-CoV2 infections (36). In this regard, the low response of older patients with low serum zinc to a 23-valent pneumococcal polysaccharide vaccination compared to those with higher zinc level (124), should be mentioned. However, zinc's role in the response to vaccination is generally discussed controversially and no data are available for vaccination against any corona virus.

Several studies indicate that there is an association between chemosensory dysfunctions and COVID-19 (125–133). Smell or taste is largely decreased, which might be a good disease biomarker (133). It was suggested that this might either be due to direct destruction of sensory cells by the virus, as ACE-2 is highly

expressed by the oral mucosa, or by viral entry into the brain and neuronal pathologies as was described for other SARS-CoV (133, 134). Zinc deficiency was related to significantly reduced taste sensitivity and impaired saliva secretion in humans and animals, which might involve zinc's importance for the action of carbonic anhydrase (135–140). Results from supplementation studies largely describe improvements in chemosensory functions (140, 141), but some studies did not find any effects (142) or even more severe disturbances when very high zinc concentrations were used (143). This is possibly due to investigating olfactory diseases of various origins, the lack of controlled trials and inclusion of observable studies. Thus, the benefits of zinc supplementation alone or in combination with common medical strategies should be tested for taste and smell diseases according to the available guidelines (144).

About 50% of patients that died of COVID-19 had bacterial or fungal co-infections (145), underlining the importance of sustaining the immune function by a sufficient zinc supply (1, 2, 36). In animal experiments it was shown that zinc restriction made mice highly susceptible to bacterial infection with *streptococcus pneumoniae* (146). As mentioned earlier, marginal zinc deficiency affects one third of the worldwide population and most subjects with COVID-19 are at risk of zinc deficiency (**Figure 1**). During physiological inflammatory responses, zinc is additionally redistributed to the tissues, resulting in serum hypozincemia (1, 65). In combination with the pre-existing suboptimal zinc supply, this will decrease serum zinc levels to critically low values and thereby significantly increase the susceptibility for co-infections with detrimental progression. In critically ill patients persistent low serum zinc was associated with recurrent sepsis and serum zinc levels were inversely correlated with mortality from sepsis (62), underlining the potential benefits of monitoring the zinc status of the patients and implementing zinc supplementation into therapy of COVID-19.

Vascular complications resulting from atherosclerosis development, microangiopathic organ failure, and venous thromboembolism were found as a major cause of death in COVID-19 patients (147–149), suggesting an important role of disease-induced coagulopathy, which, however, needs further investigation. Zinc influences thrombocyte aggregation and coagulation (150). Recently, a functional association between zinc and ROS production in platelets was described, indicating that zinc could decrease thrombus formation in a clinical context (151). Complications of SARS-CoV2 infections also include tissue damage affecting the gastrointestinal system (152), the liver (153), heart (154), nervous system (155), kidneys (156), blood vessels (149), and the skin (157). In this regard it should be mentioned that a balanced zinc homeostasis is essential for wound healing and tissue recovery after mechanical and inflammation-mediated damage (158, 159), adding more potential benefits of zinc supplementation of COVID-19 patients (**Figure 1.11**).

CONCLUSION

In this perspective, we reviewed the most important literature on the role of zinc homeostasis during viral infections, focusing on the potential benefits of zinc supplementation to prevent and treat SARS-CoV2 infections. Although data specifically on SARS-CoV2 are unfortunately still pending and randomized controlled studies have not been conducted, the enumerated evidence from the literature strongly suggests great benefits of zinc supplementation. Zinc supplementation improves the mucociliary clearance, strengthens the integrity of the epithelium, decreases viral replication, preserves antiviral immunity, attenuates the risk of hyper-inflammation, supports anti-oxidative effects and thus reduces lung damage and minimized secondary infections. Especially older subjects, patients with chronic diseases and most of the remaining COVID-19 risk groups would most likely benefit. Although studies are needed testing the effect of zinc as therapeutic option for established disease, preventive supplementation of subjects from risk groups should begin now, as zinc is a cost-efficient, globally available and simple to use option with little to no side effects. The first clinical trials on zinc supplementation alone and in combination with other drugs such as chloroquine have been registered (124, 160–162). Thus, first results and treatment regimens regarding zinc supplementation for COVID-19 risk groups and patients can be anticipated soon.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR'S NOTE

LR is a member of ZINC Net.

AUTHOR CONTRIBUTIONS

IW, BR, and LR drafted and corrected the text, table, and figure. All authors contributed to the article and approved the submitted version.

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FOI 26-3132 - Document 5

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FOI 26-3132 - Document 5

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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FOI 26-3132 - Document 5

Ther Adv Respir Dis

2020, Vol. 14: 1–5

DOI: 10.1177/1753466620951053

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Doxycycline treatment of high-risk COVID-19-positive patients with comorbid pulmonary disease

Paul A. Yates, Steven A. Newman, Lauren J. Oshry, Robert H. Glassman, Ashton M. Leone and Elias Reichel

***Abstract*:** Infection with novel SARS-CoV-2 carries significant morbidity and mortality in patients with pulmonary compromise, such as lung cancer, autoimmune disease, and pneumonia. For early stages of mild to moderate disease, care is entirely supportive. Antiviral drugs such as remdesivir may be of some benefit but are reserved for severe cases given limited availability and potential toxicity. Repurposing of safer, established medications that may have antiviral activity is a possible approach for treatment of earlier-stage disease. Tetracycline and its derivatives (e.g. doxycycline and minocycline) are nontraditional antibiotics with a well-established safety profile, potential efficacy against viral pathogens such as dengue fever and chikungunya, and may regulate pathways important in initial infection, replication, and systemic response to SARS-CoV-2. We present a series of four high-risk, symptomatic, COVID-19$^+$ patients, with known pulmonary disease, treated with doxycycline with subsequent rapid clinical improvement. No safety issues were noted with use of doxycycline. Doxycycline is an attractive candidate as a repurposed drug in the treatment of COVID-19 infection, with an established safety profile, strong preclinical rationale, and compelling initial clinical experience described here.

The reviews of this paper are available via the supplemental material section.

Keywords: COVID-19, doxycycline, pulmonary disease, SARS-CoV-2, therapeutic

Received: 24 June 2020; revised manuscript accepted: 7 July 2020.

Introduction

SARS-CoV-2 is a newly emerged coronavirus for which there are limited therapeutic options and no available vaccine.[1] Patients who test positive are advised to self-quarantine, with supportive care including rest and fluid intake.[1] Hospitalization is indicated for severe disease and may be considered for patients with moderate disease with high-risk features, including immunocompromised states (e.g. cancer treatment, chronic lung disease, diabetes, severe obesity).[1] Mortality in patients with lung cancer and COVID-19 infection is high; 33% in a recently reported international series.[2]

There is significant interest in repurposing existing, approved medications for treatment of COVID-19. Tetracycline and its derivatives (e.g. doxycycline) may offer a rational and safe treatment for COVID-19$^+$ patients. Doxycycline is commonly dosed at 100 mg twice daily to treat bacterial infections and dermatologic conditions (e.g. acne vulgaris and rosacea).[3] Tetracyclines have both antiviral and anti-inflammatory properties that may ameliorate the response to viral infection.[3–6] Clinical studies indicate efficacy of doxycycline against dengue and chikungunya viral infections.[4,5] In addition, doxycycline may affect several pathways regulating viral replication.[7–9] These pleiotropic features of tetracyclines likely result from actions on nontraditional, antibiotic molecular pathways.[3]

Doxycycline has several potential mechanisms of action through which it may prevent or ameliorate

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FOI 26-3132 - Document 5

the effects of COVID-19 infection. Doxycycline is well known to inhibit metalloproteinases (MMPs), in particular MMP-9, which is likely required for initial viral entry into the cell.[3] Doxycycline inhibits interleukin (IL)-6, with both IL-6 and MMPs key regulators of the 'cytokine storm' often associated with severe viral pneumonitis.[10–12] Doxycycline is an established ionophore, helping transport zinc intracellularly, with increased cellular concentrations of zinc shown *in vitro* to inhibit coronavirus replication.[3,13] Doxycycline inhibits nuclear factor (NF)-κB[3,14], which may lower risk of viral entry due to direct inhibition of DPP4 cell surface receptor[15] and diminish a hyperactive immune response following infection. Nonantimicrobial, low-dose doxycycline has been found *in vivo* to inhibit expression of CD147/EMMPRIN,[16] which may be necessary for SARS-CoV-2 entry into T lymphocytes.[16,17] Structural analysis demonstrates that doxycycline has the potential to inhibit papain-like proteinase (PLpro) and 3C-like main protease (3CLpro), viral proteins both essential to viral replication and lifecycle.[9]

In view of the above considerations, we report a series of four patients with COVID-19 infection and known high-risk pulmonary disease who were placed on standard doses of doxycycline for a course between 5 and 14 days. Patients were initiated on therapy with doxycycline after discussion of potential risks and benefits and after informed consent was obtained. There was no concomitant use of any other antibiotics, antiviral agents, antimalarial drugs, zinc, or any supplements postulated to be beneficial in therapy for SARS-CoV-2 in these four patients.

Case 1

A 48-year-old black woman, never smoker, with stage IV epidermal growth factor receptor (EGFR) mutated adenocarcinoma of lung, diagnosed in August 2018, was treated with osimertinib since November 2018 with response in all sites of disease. Other medications included loperamide and doxycycline, as needed for EGFR inhibitor-related diarrhea and rash, respectively. She worked as a nursing assistant. On 6 April 2020 she reported fever of 101° F, headache, anorexia and anosmia. On examination: temperature 101° F, pulse 102, blood pressure 115/71, O_2 sat 98% on room air. She was noted to have mild cough with normal respiratory effort. Laboratory tests revealed white blood cell count of 2.7, with lymphopenia, similar to prior recent determinations; C-reactive protein (CRP) 10.6 (0–5 mg/l), creatine kinase (CK) 2996 (46–179 U/l), troponin <0.006 (negative), aspartate aminotransferase 105 (13–39 U/l), creatinine 1.3 (05–1.1) mg/dl. Chest X-ray showed chronic stable right retrocardiac midlung opacities with no new focal consolidation or effusion. The following day her swab returned positive for SARS-CoV-2. As she had declined hospitalization, she was instructed to discontinue osimertinib but to start doxycycline initially 200 mg followed by 100 mg daily for 5 days. She noted improvement in all symptoms within 5 days.

Case 2

A 71-year-old white male physician with a 9 year history of pulmonary sarcoidosis, treated with intermittent steroids, noted new intermittent cough and one episode of diarrhea in early April 2020. Nasopharyngeal swab was positive for SARS-CoV-2. No additional evaluation was performed. The patient was started on doxycycline 100 mg orally twice a day and self-monitored pulse oximetry at home. Revisit to the emergency department was prompted by transient drop in oxygen saturation to 88% on day six. His temperature was 100.4° F, chest X-ray showed evidence of his chronic lower lung changes attributed to sarcoidosis, O_2 sat was 96%. The patient returned home without supplemental oxygen and remained on doxycycline for 10 days. Fever resolved and he remained asymptomatic. After 3 weeks retesting was negative for SARS-CoV-2 and he returned to work.

Case 3

A 40-year-old Asian woman, never smoker, with stage IV EGFR mutated lung adenocarcinoma, on osimertinib since 13 June 2018, also treated with stereotactic radiation to right lower lobe primary lesion in July 2019, presented to an urgent care clinic with 4 days of anosmia, mild cough, and mild dyspnea. She worked as a personal care assistant for elderly patients at home. Nasopharyngeal swab testing was positive for SARS-CoV-2 RNA. The patient continued to take osimertinib and doxycycline 100 mg twice daily (previously prescribed for EGFR-I related

rash). Symptoms improved within 1 week. Repeat nasopharyngeal swab testing on 9 May 2020 was negative for SARS-CoV-2 RNA.

Case 4

An 88-year-old white man, with several comorbidities including sick sinus syndrome, cardiovascular disease, lacunar strokes, mild obstructive lung disease, and chronic indwelling bladder catheter, was admitted from a Veterans Nursing Home with fever to 102°F, severe dry cough, weakness, and dyspnea. Chest X-ray showed bilateral diffuse infiltrates. Laboratory tests were notable for nasopharyngeal swab positive for SARS-CoV-2 RNA, CRP 11, mild hypoxia with O_2 sat 89–92%. Ceftriaxone, azithromycin and 2 liters of nasal prong O_2 were begun. His initial hospital course showed progression of his pulmonary symptomatology with decreasing O_2 saturation requiring 4 liters of nasal prong O_2 by day 3, with CRP increase to 13. On day 4 ceftriaxone was discontinued and azithromycin was replaced with doxycycline 100 mg twice daily. His pulmonary course quickly improved alongside decreasing CRP. By day 8, he no longer required oxygen and his CRP was 1.0. He never received any antiviral, IL-6 antibody, nor convalescent plasma. He was discharged on day 28 after his lung infiltrates resolved on chest X-ray after 14 days of doxycycline therapy.

Discussion

These reported cases are the first to provide a correlative relationship between doxycycline and potential reduction of severity of symptoms in COVID-19$^+$ patients. It is difficult to establish a definitive causal link between use of doxycycline and accelerated recovery or decreased morbidity from SARS-CoV-2 infection in a small case series. We believe this study provides a rationale for initiating larger controlled trials evaluating the use of doxycycline in COVID-19$^+$ patients.

The patients we report had a number of high-risk features, predictive of severe disease and increased risk of mortality. Patients one and four had advanced lung cancer on treatment, a group for whom mortality rates with COVID-19 infection is in excess of 33%.[2] Elevated inflammatory markers, including CRP, transaminases, and CK (as in cases 1 and 4) are also associated with worse outcomes.[18] Advanced age and diffuse bilateral infiltrates (case 4), are also predictors of high morbidity and mortality.[19] Case 4 demonstrated a temporal relationship between administration of doxycycline and clinical improvement, after several days of worsening on azithromycin. Patients with active pulmonary sarcoidosis, as in case 2, are also at greater risk of complications from COVID-19 due to immunologic dysfunction and dysregulation.[20] The described patients were at a significant increased risk from COVID-19 morbidity and mortality. All improved, with one showing resolution of bilateral pulmonary infiltrates. None progressed to severe disease.

A standard antimicrobial dose of doxycycline (100 mg twice daily or daily) was administered to the four patients in this case series. Doxycycline is unique among the many tetracycline derivatives, in that it has a clear dosing split between its cellular cytotoxic/antimicrobial effects (>100 mg) and its potential anti-inflammatory/antiviral/pro-ionophoric effects (40 mg).[3,4,7,8,12] This may be an important consideration for low-dose doxycycline (20 mg twice daily) as a potential prophylactic treatment against COVID-19 infection.

Potential efficacy of doxycycline against COVID-19 may be due to pleiotropic effects against the general pathways involved in viral infection, replication, and associated over-exuberant inflammatory response, with associated angiogenic effects.[3] Doxycycline has no known direct specificity for inhibition of SARS-CoV-2. Doxycycline likely has activity against known coreceptors DPP4/CD26, through demonstrated inhibition of NF-κB,[14,15] and coreceptor CD147/EMMPRIN, necessary for entry of SARS-CoV into T lymphocytes, even at submicrobial doses.[16,17] DPP4 appears to have a binding site for NF-κB and inhibition of NF-κB decreases DPP4 expression.[15] DPP4 demonstrates increased expression in older patients and those with diabetes or pulmonary disease, and may account in part for increased morbidity and mortality to COVID-19.[8] Finally, MMP-9 also appears to be required for the virus to traverse the cell wall for infection.[10,11]

Once COVID-19 has infected the cell, structural analysis indicates doxycycline may be able to bind to PLpro, which is responsible for proteolytic cleavage of the replicase polyprotein that releases nonstructural proteins 1, 2 and 3 (Nsp1, Nsp2 and Nsp3), essential for viral replication.[9] It is similarly predicted to bind to 3CLpro or

FOI 26-3132 - Document 5

Nsp5, which is cleaved from the polyproteins, causing further cleavage of Nsp4-16, mediating maturation of Nsps essential in the virus lifecycle.[9] Doxycycline is an ionophore that binds divalent cations (including zinc) to facilitate cell membrane transport, and thereby increase zinc concentration in the cell.[3,13] Elevated zinc has an inhibitory effect on the replication of SARS-CoV-2.[13] The presumed mechanisms are through inhibition of proper processing of replicase proteins and RNA-dependent RNA polymerase (RdRp) activity.[13]

Lastly, doxycycline may help prevent the 'cytokine storm' following COVID-19 infection presumed to be due to hyperactivity of the immune response to SARS-CoV-2. A primary driver of the anti-inflammatory activity of doxycycline is secondary to its direct inhibition of NF-κB.[14] NF-κB directly regulates IL-6 expression, which is a key driver of the cytokine storm.[21] Doxycycline may also precipitate apoptosis of senescent epithelial cells,[8] which have known increased expression of DPP4/CD26, likely exhibiting increased viral replication in these cells which leads to induction of the cytokine storm.

Conclusion

In summary, we present reports of four patients at high risk for morbidity and mortality from SARS-CoV-2 infection who experienced improvement and/or mild clinical course in association with doxycycline. Despite the safety of doxycycline, we would not recommend general use of this medication for treatment of COVID-19^{+} patients, except under direct physician guidance and monitoring. A larger randomized controlled trial should be considered.

Author contribution(s)

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Steven A. Newman: Data curation; Investigation; Writing-original draft.

Lauren J. Oshry: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

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Supplemental material

The reviews of this paper are available via the supplemental material section.

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FAST TRACK

Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-Cov-2) outside of Wuhan, China: retrospective case series

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Additional material is published online only. To view please visit the journal online.

Cite this as: *BMJ* 2020;368:m606
http://dx.doi.org/10.1136/bmj.m606

Accepted: 13 February 2020

ABSTRACT

OBJECTIVE

To study the clinical characteristics of patients in Zhejiang province, China, infected with the 2019 severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) responsible for coronavirus disease 2019 (covid-2019).

DESIGN

Retrospective case series.

SETTING

Seven hospitals in Zhejiang province, China.

PARTICIPANTS

62 patients admitted to hospital with laboratory confirmed SARS-Cov-2 infection. Data were collected from 10 January 2020 to 26 January 2020.

MAIN OUTCOME MEASURES

Clinical data, collected using a standardised case report form, such as temperature, history of exposure, incubation period. If information was not clear, the working group in Hangzhou contacted the doctor responsible for treating the patient for clarification.

RESULTS

Of the 62 patients studied (median age 41 years), only one was admitted to an intensive care unit, and no patients died during the study. According to research, none of the infected patients in Zhejiang province were ever exposed to the Huanan seafood market, the original source of the virus; all studied cases were infected by human to human transmission. The most common symptoms at onset of illness were fever in 48 (77%) patients, cough in 50 (81%), expectoration in 35 (56%), headache in 21 (34%), myalgia or fatigue in 32 (52%), diarrhoea in 3 (8%), and haemoptysis in 2 (3%). Only two patients (3%) developed shortness of breath on admission. The median time from exposure to onset of illness was 4 days (interquartile range 3-5 days), and from onset of symptoms to first hospital admission was 2 (1-4) days.

CONCLUSION

As of early February 2020, compared with patients initially infected with SARS-Cov-2 in Wuhan, the symptoms of patients in Zhejiang province are relatively mild.

WHAT IS ALREADY KNOWN ON THIS TOPIC

As of 8 February 2020, coronavirus disease 2019 (covid-19) caused by the 2019 severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) has been responsible for more than 30 000 infections and 700 deaths globally, and the numbers are still increasing rapidly

The reported mortality from infection with SARS-Cov-2 in Wuhan, China reached 11-15%, which was inconsistent with what was observed outside of Hubei province

The epidemiological and clinical characteristics of patients outside of Wuhan have not been described, especially in Zhejiang province, where by 8 February 2020 more than 1000 people were diagnosed as having covid-19

WHAT THIS STUDY ADDS

In patients infected with SARS-Cov-2 in Zhejiang province, the median time from exposure to onset of illness was 4 days (interquartile range 3-5 days) and from onset of symptoms to first hospital admission was 2 (1-4) days

The most common symptoms were fever, cough, expectoration, headache, myalgia or fatigue, diarrhoea, and haemoptysis, and only two patients developed shortness of breath on admission

At present, compared with the initial patients infected in Wuhan, the symptoms of patients in Zhejiang province are relatively mild

Introduction

In December 2019 a group of patients with pneumonia of unknown cause were confirmed to be infected with a novel coronavirus, known as 2019-nCoV, in Wuhan, Hubei province, China, which had previously not been detected in humans or animals.[1] Epidemiological evidence suggested that most of these patients had visited a local seafood market in Wuhan[2] and that the gene sequence of the virus obtained from these patients was highly similar to that identified in bats.[3] The virus was subsequently renamed SARS-Cov-2 as it is similar to the coronavirus responsible for severe acute respiratory syndrome (SARS-CoV), a member of the subgenus Sarbecovirus (Beta-CoV lineage B), with which it shares more than 79% of its sequence, but it is more distant to the coronavirus responsible for Middle East respiratory syndrome (MERS-CoV), a member of the Merbecovirus subgenus (only 50% homology with SARS-Cov-2). All these viruses are categorised within the same genus of the subfamily Orthocoronavirinae within the family Coronaviridae.[4-7] Some researchers have found that SARS-Cov-2 has strong affinity to human respiratory receptors,[8] suggesting a potential threat to global public health.

Initially, the first confirmed cases were nearly all related to the Huanan seafood market (closed on 1 January 2020) and were concentrated in Wuhan.[9] Coronavirus disease 2019 (covid-19) soon drew global attention because of the rapidly increasing numbers of new cases.[2] The new type of coronavirus infection was believed to have been transmitted from animals, and by January 2020 it was suspected that the initially

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FOI 26-3132 - Document 5

affected patients had been infected with the virus through human to human transmission.[10] Since January 2020 the spread of covid-19 has escalated and the virus has extended rapidly to most parts of China as well as to other countries. As of 8 February 2020 a confirmed 37 589 people have been infected with SARS-Cov-2 globally, including 302 people across 24 other countries.[11] These figures are updated daily and are expected to increase further.

Despite the increasing number of confirmed cases, the clinical investigation of patients was insufficient. A previous study reported the clinical characteristics of the first 41 infected patients in the greater Wuhan area, contributing to an understanding of the epidemiological, clinical, laboratory, and radiological characteristics and treatment and clinical outcomes of those patients.[9] A second study found a familial cluster of SARS-Cov-2, clearly suggesting human to human transmission in family homes and hospitals and showing that spread of the virus between cities is possible.[10] In late January 2020 large numbers of people across China were returning to their home towns after visiting Wuhan for the Chinese lunar new year. This weeklong holiday accounts for the largest mass movement of people worldwide each year. Consequently, over time more patients are expected to emerge across China and perhaps the world. We found that the characteristics of patients outside of Wuhan differed from those initially reported in patients in Wuhan.[10]

We describe the clinical characteristics and laboratory findings of patients in Zhejiang province infected with SARS-Cov-2 to provide an insight into the prevention and treatment of covid-19 across China and elsewhere.

Methods

Data sources

We conducted a retrospective study focusing on the clinical characteristics of confirmed cases of covid-19 in Zhejiang province from 10 January 2020 to 26 January 2020. Since the outbreak of covid-19, strict precautionary measures have been implemented in Zhejiang province, including the creation of fever clinics that exclusively receive patients with suspected SARS-Cov-2 infection, defined as presenting with a fever or any respiratory symptoms, including dry cough, and especially in those with a history of travel to Wuhan or exposure to infected people within two weeks before the onset of illness since January 2020. Case definitions of confirmed human infection with SARS-Cov-2 are in accordance with the interim guidance from the World Health Organization.[1] Only patients with a laboratory confirmed infection were enrolled in this study. We collected data on 62 patients admitted to hospital with laboratory confirmed SARS-Cov-2 infection in seven designated tertiary hospitals in Zhejiang province (see supplementary file for further details). Information was collected on dates of illness onset, visits to clinical facilities, and hospital admissions. Epidemiological data were collected through brief interviews with each patient. Several investigators interviewed each patient to collect exposure histories during the two weeks before illness onset, including the dates and times of close contact (gathering, living, or working together) with individuals from Wuhan with confirmed or suspected SARS-Cov-2 infection. The incubation period was defined as the time from exposure to the onset of illness, which was estimated among patients who could provide the exact date of close contact with individuals from Wuhan with confirmed or suspected SARS-Cov-2 infection. We also investigated the possibility of familial clusters—that is, index patients who travelled to Wuhan and then infected others in their families.

We extracted the medical records of patients and sent these to the data collection centre in Hangzhou. A team of doctors who had been treating patients with covid-19 collected and reviewed the data. Because of the urgent need to collect data on this emerging pathogen, the requirement for informed consent was waived. We used a standardised case report form to collect clinical data. If information was not clear, the working group in Hangzhou contacted the doctor responsible for the treatment of the patient for clarification.

Laboratory confirmation and treatment

Sputum and throat swab specimens collected from all patients at admission were tested by real time polymerase chain reaction for SARS-Cov-2 RNA within three hours. Laboratory confirmation of the virus was performed using real time reverse transcription polymerase chain reaction.[9] Virus detection was repeated twice every 24 hours.

Laboratory tests were conducted at admission, including a complete blood count, serum biochemistry, and identification of other respiratory pathogens such as influenza A virus (H1N1, H3N2, H7N9), influenza B virus, respiratory syncytial virus, parainfluenza virus, and adenovirus. Most patients received antiviral treatment with interferon alpha inhalation (50 µg twice daily), lopinavir and ritonavir (400 mg twice daily and 100 mg twice daily, respectively), and arbidol (200 mg three times daily). Patients received treatment with corticosteroid (40-80 mg/day) and gamma globulin (15-20 g/day) for 3-5 days when their resting respiratory rate was more than 30 per minute, or oxygen saturation was below 93% without oxygen, or multiple pulmonary lobes showed more than 50% progression of disease in 48 hours on imaging. Patients also received treatment with probiotics in most cases. Quinolones and second generation beta lactams (oral and intravenous) were administered if fever lasted for more than seven days or C reactive protein levels were 30 mg/L or more (normal range 0-8 mg/L). Patients suspected of being infected with SARS-Cov-2 were discharged from hospital once the results of two real time reverse transcription polymerase chain reaction tests taken 24 hours apart were negative for SARS-Cov-2 antigens.

Statistical analysis

As a previous study[9] has shown that patients' condition worsens on the 10th day after illness onset, we divided the cohort into patients with symptoms for more than

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doi: 10.1136/bmj.m606 | *BMJ* 2020;368:m606 | the bmj

10 days and those with symptoms for less than 10 days. We summarised continuous variables as either means and standard deviations or medians with interquartile ranges. For categorical variables, we calculated the percentages of patients in each category. All analyses were done with SPSS software, version 22.0.

Patient and public involvement

This was a retrospective case series study and no patients were involved in the study design, setting the research questions, or the outcome measures directly. No patients were asked to advise on interpretation or writing up of results.

Results

Epidemiological characteristics

By 26 January 2020, clinical data were collected on 62 patients in Zhejiang province with laboratory confirmed SARS-Cov-2 infection. Twenty five (40%) of the patients were aged 19-40 years, 33 (53%) were aged 41-65 years, 2 (3%) were aged 10 and 11 years, and 2 (3%) were aged 65 years and older. The median age was 41 years (interquartile range 32-52 years; table 1). As of 26 January 2020, more than half of the 62 patients (35, 56%) were men. No patients had a history of exposure to the Huanan seafood market and all 62 patients had been exposed to individuals with confirmed SARS-Cov-2 infection. Among the 62 patients, 23 (37%) resided in Wuhan and the remaining 39 (63%) had made short term trips to Wuhan before illness onset. Fifty six (90%) patients could provide the exact date of close contact with someone with confirmed or suspected SARS-Cov-2 infection.

Of the 33 patients with symptoms for more than 10 days after illness onset, 10 (30%) were aged 19-40 years, 22 (67%) were aged 41-65 years, and 1 (3%) was older than 65 years. The median age of patients was 45 years (interquartile range 37-54 years; table 1).

Clinical features

Twenty of the 62 patients (32%) had underlying diseases—seven (11%) had liver disease, five (8%)

Table 1 | Personal and clinical characteristics of 62 patients with coronavirus disease 2019 (covid-19) in Zhejiang province, China. Values are numbers (percentages) unless stated otherwise

Characteristics	All patients (n=62)	Time since symptom onset	
		>10 days (n=33)	≤10 days (n=29)
Median (interquartile) age (years)	41 (32-52)	45 (37-54)	39 (31-50)
Age groups (years):			
≤18	2 (3)	0 (0)	2 (7)
19-40	25 (40)	10 (30)	15 (52)
41-65	33 (53)	22 (67)	11 (38)
≥66	2 (3)	1 (3)	1 (3)
Sex:			
Male	35 (56)	19 (58)	16 (55)
Female	27 (44)	14 (42)	13 (45)
Coexisting conditions:			
Any	20 (32)	13 (39)	7 (24)
Hypertension	5 (8)	4 (12)	1 (3)
Diabetes	1 (2)	1 (3)	0 (0)
Chronic obstructive pulmonary disease	1 (2)	1 (3)	0 (0)
Cerebrovascular disease	1 (2)	1 (3)	0 (0)
Renal diseases	1 (2)	0 (0)	1 (3)
Liver disease	7 (11)	4 (12)	3 (10)
Exposure history in Wuhan >2 weeks:			
Yes	23 (37)	9 (27)	14 (48)
No	39 (63)	24 (73)	15 (52)
Familial cluster	21 (34)	9 (27)	12 (41)
Fever	48 (77)	24 (73)	24 (83)
Highest temperature (°C):			
<37.3	14 (23)	9 (27)	5 (17)
37.3-38.0	22 (35)	10 (30)	12 (41)
38.01-39.0	18 (29)	9 (27.3)	9 (31)
>39.0	8 (13)	5 (15.	3 (10)
Respiratory rate >24 breaths per min	2 (3)	1 (3)	1 (3)
Arterial oxygen pressure (mm Hg)	90 (80-105) (n=51)	85 (76-97) (n=28)	100 (86-125) (n=23)
Oxygenation index	379 (292-448) (n=51)	331 (278-422) (n=28)	409 (345-479) (n=23)
Mean arterial pressure (mm Hg)	97 (87-106)	95 (86-105)	99 (88-107)
Cough	50 (81)	27 (82)	23 (79)
Myalgia or fatigue	32 (52)	19 (58)	13 (45)
Expectoration	35 (56)	19 (58)	16 (55)
Haemoptysis	2 (3)	2 (6)	0 (0)
Headache	21 (34)	15 (45)	6 (21)
Diarrhoea	3 (8)	3 (9)	0 (0)
Incubation period (days)	4 (3-5) (n=56)	3 (3-4) (n=29)	5 (4-7) (n=27)
Time from illness onset to first hospital admission (days)	2.0 (1.0-4.3)	6.5 (5.0-9.0)	2 (1-2)

Percentages do not total 100% owing to missing data.

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FOI 26-3132 - Document 5

Table 2 | Laboratory and chest radiography findings in patients with coronavirus disease 2019 (covid-19) on admission to hospital in Zhejiang province, China. Values are medians (interquartile ranges) unless stated otherwise

Variables	All patients (n=62)	Time since symptom onset		Normal range
		>10 days (n=33)	≤10 days (n=29)	
White blood cell count (×10^9/L)	4.7 (3.5-5.8)	4.5 (3.1-6.1)	4.9(3.9-5.7)	4-10
White blood cell count (×10^9/L) (No (%)):				
<4	19 (31)	13 (39)	6 (21)	
4-10	42 (68)	20 (61)	22 (76)	
>10	1 (2)	0 (0)	1 (3)	
Neutrophil count (×10^9/L)	2.9 (2.0-3.7)	2.8 (1.7-3.9)	2.9 (2.3-3.7)	2-7
Lymphocyte count (×10^9/L)	1.0 (0.8-1.5)	1.0 (0.7-1.4)	1 (0.9-1.5)	0.8-4
Lymphocyte count (×10^9/L) (No (%)):				
<1.0	26 (42)	15 (45)	11 (38)	
≥1.0	36 (58)	18 (55)	18 (62)	
Haemoglobin (g/L)	137.0 (128.8-152.3)	137.5 (129.0-149.5)	136.5 (127.3-153.8)	113-151
Platelet count (×10^9/L):	176.0 (135.8-215.5)	172.5 (128.8-202.8)	186 (149-228)	83-303
<100	3 (5)	3 (9)	0 (0)	
≥100	59 (95)	30 (91)	29 (100)	
D-dimer (mg/L)	0.2 (0.2-0.5)	0.2 (0.2-0.6)	0.2 (0.2-0.4)	0-0.7
Alanine aminotransferase (U/L)	22 (14-34)	22.5 (13.6-35.1)	22.0 (14.1-34.5)	7-40
Aspartate aminotransferase (U/L)	26 (20-32)	27.5 (18.5-34.7)	25.0 (21.5-29)	13-35
Aspartate aminotransferase (U/L) (No (%)):				
<40	52 (84)	28 (84.8)	24 (82.8)	
≥40	10 (16.1)	5 (15)	5 (17)	
Potassium (mmol/L)	3.7 (3.5-3.9)	3.6 (3.5-3.9)	3.8 (3.4-4)	3.5-5.3
Sodium (mmol/L)	139 (127-141)	138.6 (136.9-140.6)	139 (137.1-141.5)	137-147
Creatinine (μmol/L)	72.0 (61.0-84.0)	71.5 (61.0-82.2)	71.4 (60.3-90.5)	41-73
Creatinine (μmol/L) (No (%)):				
≤133	59 (95)	33 (100)	26 (90)	
>133	3 (5)	0 (0)	3 (10)	
Creatinine kinase (U/L):	69.0 (40.5-101.0)	60.0 (40.0-106.8)	71.4 (60.3-90.5)	40-200
Creatinine kinase (U/L) (No (%)):				
≤185	57 (92)	31 (94)	26 (90)	
>185	5 (8)	2 (6)	3 (10)	
Lactate dehydrogenase (U/L):	205.0 (184.0-260.5)	233.5 (198.0-312.3)	194.5 (166.3-213.8)	120-250
Lactate dehydrogenase (U/L) (No (%)):				
≤245	45 (73)	19 (58)	26 (90)	
>245	17 (27)	14 (42)	3 (10)	
Procalcitonin (ng/mL):	0.04 (0.03-0.06)	0.04 (0.035-0.06)	0.04 (0.025-0.06)	0-0.05
Procalcitonin (ng/mL) (No (%)):				
<0.1	55 (89)	31 (94)	24 (83)	
≥0.1	7 (11)	2 (6)	5 (17)	
Bilateral involvement on chest radiographs	52 (84)	32 (97)	20 (69)	
Pneumonia	61 (98)	33 (100)	28 (97)	

Percentages do not total 100% owing to missing data.

had hypertension, and one (2%) each had chronic obstructive pulmonary disease, diabetes, renal disease, and cerebrovascular disease. Twenty one of the 62 patients (34%) were associated with familial clusters.

Of the 33 patients with symptoms for more than 10 days after illness onset, 13 (39%) had underlying diseases: four (12%) patients had liver disease, four (12%) had hypertension, and one each had chronic obstructive pulmonary disease (3%), diabetes (3%), and cerebrovascular disease (3%). Among 56 patients who could provide the exact date of close contact with someone with confirmed or suspected SARS-Cov-2 infection, the median incubation period from exposure to symptoms was 4 days (interquartile range 3-5 days). The median time from onset of symptoms to first hospital admission was 2.0 (1.0-4.3) days. The most common symptoms at illness onset were fever (48, 77%), cough (50, 81%), expectoration (35, 56%), headache (21, 34%), myalgia or fatigue (32, 52%), diarrhoea (3, 8%), and haemoptysis (2, 3%). Only two (3%) patients developed shortness of breath. Among the 33 patients who had symptoms for more than 10 days after illness onset, the median incubation period from exposure to symptoms was 3 days (interquartile range 3-4 days). The median time from onset of symptoms to first hospital admission was 6.5 (5.0-9.0) days. The most common symptoms at onset of illness were cough (27, 82%), fever (24, 73%), expectoration (19, 58%), myalgia or fatigue (19, 58%), headache (15, 45%), diarrhoea (3, 9%), and haemoptysis (2, 6%). Only one (3%) patient developed shortness of breath.

On admission, the blood counts of 19 of the 62 (31%) patients showed leucopenia (white blood cell count <4×10^9/L) and 26 (42%) showed lymphopenia (lymphocyte count <1.0×10^9/L; table 2). The D-dimer levels were within normal range (median 0.2 mg/L (interquartile range 0.2-0.5 mg/L). Levels of aspartate aminotransferase increased in 10 (16%) patients.

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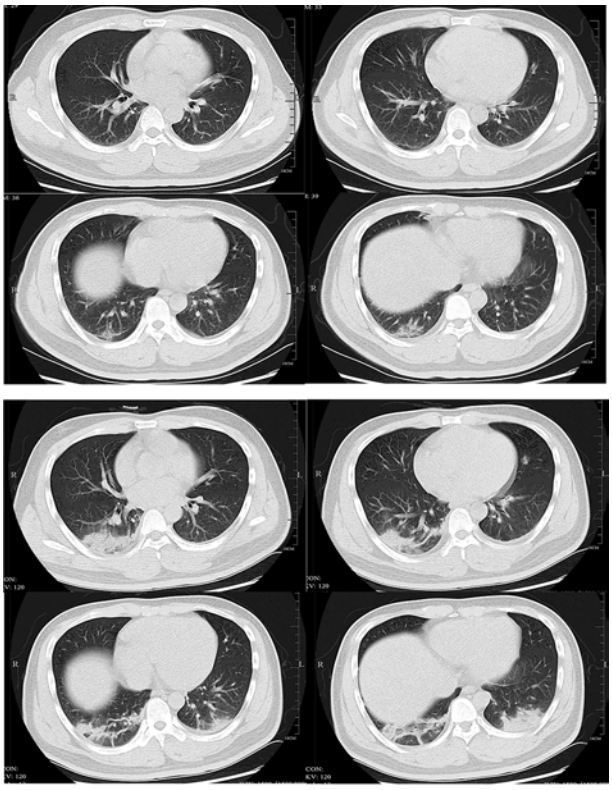


Fig 1 | Transverse chest computed tomograms from a 32 year old man, showing ground glass opacity and consolidation of lower lobe of right lung near the pleura on day 1 after symptom onset (top panel), and bilateral ground glass opacity and consolidation on day 7 after symptom onset (bottom panel)

Fifty five (89%) patients had normal serum levels of procalcitonin (<0.1 ng/mL). Abnormalities on chest computed tomograms or radiographs were detected among all of the patients except for one. Fifty two (84%) patients showed bilateral involvement on chest radiographs (table 2). Typical chest computed tomography findings of infected patients on admission were bilateral or multiple lobular or subsegmental areas of consolidation or bilateral ground glass opacity (fig 1). Only one patient did not have pneumonia. Of the 62 patients, only one was transferred to an intensive care unit for acute respiratory distress syndrome and received mechanical ventilation (table 3). Fifty five (89%) patients received antiviral treatment, 28 (45%) were given empirical antibiotic treatment, and 16 (26%) were given systematic corticosteroid and gamma globulin treatment. At this point, one (2%) patient had been discharged and no patients had died. Fitness for discharge was based on abatement of fever for at least three days, with improved evidence on chest radiography and viral clearance in samples from the lower respiratory tract.

Discussion

As of 8 February 2020, more than 30 000 laboratory confirmed cases of infection with the novel coronavirus (SARS-Cov-19) were reported in China.[12] The number of infections is increasing rapidly. It is possible that an even greater number of infected patients exist without a diagnosis because their symptoms were less severe and because of the incubation period. Thousands of patients with suspected SARS-Cov-2 infection could eventually receive a diagnosis. The clinical features of early cases of covid-19 in Wuhan were not the same as those in other areas of China. According to our data, none of the infected patients in Zhejiang province had been exposed to the Huanan seafood market, and as the number of familial clusters in infected patients in our study is large, this might suggest human to human transmission. This finding is also consistent with a published article.[10] Further detailed investigations should aim to ascertain the exact mode of transmission.

Most of the infected individuals in Zhejiang province were male patients, but the age range of patients is large as SARS-Cov-2 also infected children and those older than 65 years.[9 13] No major differences were found between the initial clinical symptoms of patients in Zhejiang province and those in Wuhan. Most of

Table 3 | Treatments and outcomes in patients with coronavirus disease 2019 (covid-19) on admission to hospital in Zhejiang province, China. Values are numbers (percentages) of patients

		Time since symptom onset	
Treatments and outcomes	All patients (n=62)	>10 days (n=33)	≤10 days (n=29)
Admission to intensive care unit	1 (2)	1 (3)	0 (0)
Acute respiratory distress syndrome	1 (2)	1 (3)	0 (0)
Treatment			
Antiviral treatment	55 (89)	31 (94)	24 (83)
Interferon alpha inhalation	8 (13)	4 (12)	4 (14)
Lopinavir/ritonavir	4 (6)	3 (9)	1 (3)
Arbidol+interferon alpha inhalation	1 (2)	0 (0)	1 (3)
Lopinavir/ritonavir+interferon alpha inhalation	21 (34)	7 (21)	14 (48)
Arbidol+lopinavir/ritonavir	17 (28)	13 (39)	4 (14)
Arbidol+lopinavir/ritonavir+interferon alpha inhalation	4 (6)	4 (12)	0 (0)
Antibiotics	28 (45)	16 (48)	12 (41)
Corticosteroid and gamma globulin	16 (26)	11 (33)	5 (17)
Prognosis			
Hospital admission	61 (98)	32 (97)	29 (100)
Discharge	1 (2)	1 (3)	0 (0)
Death	0	0	0

Percentages do not total 100% owing to missing data.

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the patients in Zhejiang province, however, had mild to moderate symptoms, and only a small portion of them had dyspnoea. Only one patient developed acute respiratory distress syndrome and was admitted to an intensive care unit. The laboratory test results showed that the patients also experienced mild illness. There were fewer patients with abnormal renal function and lactate dehydrogenase and procalcitonin levels. Through media and national advocacy, patients with fever, cough, expectoration, and other upper respiratory tract symptoms were asked to go to hospital at an early stage. Even those who had contact with other patients, or patients with suspected infection were asked to go to hospital. We also analysed patients with symptoms for more than 10 days after illness onset. We found that the clinical features of patients with symptoms for longer than 10 days in Zhejiang province were less severe than those of the primary infected patients from Wuhan.[9 13] This phenomenon was also apparent during the transmission of MERS-CoV. The global case mortality of MERS-CoV was about 40%, whereas the mortality from second generation MERS-CoV was about 20%.[14 15]

Patients in the two cohorts received antiviral treatment, but the types of drugs used varied between patients. Treatment with lopinavir and ritonavir were reported to have the potential to treat SARS infections,[16] and we suppose this treatment might be a beneficial part of the treatment for covid-19. The rate of antibiotic and corticosteroid use was different. Less than half the patients in Zhejiang province received antibiotics. Whether the use of antivirals, antibiotics, or steroids affects the prognosis of patients remains unknown.

Given that most infections in Zhejiang province were in patients who had no direct contact with the original site of outbreak, our findings provide valuable information in the understanding of the clinical features of covid-19, as the number of people with confirmed disease continues to increase rapidly. Our study population might represent most of the clinical characteristics of infected patients since January 2020. Further containment proposals should be implemented by the Chinese government, such as preventing people from Wuhan having contact with those elsewhere, banning gatherings of more than 100 people, conducting daily public-wide educational campaigns on precautionary measures against exposure to SARS-Cov-2, encouraging people to cancel traditional family gatherings such as during the Chinese lunar new year, and extending the Chinese lunar new year holiday to prevent large scale spread.

Limitations of this study

Our study has several limitations. Firstly, only 62 patients were included. A large number of patients were continually being admitted to hospital as data were being collected, and thus we obtained data on most but not all of the patients with laboratory confirmed infection in Zhejiang province during the study period. Secondly, as the patients were only from Zhejiang province, it might be that more clinical features related to covid-19 will be identified. Thirdly, at the time of study submission, most patients had not been discharged, so we are unable to estimate either the case fatality rate or the predictors of fatality. Moreover, the time since illness onset in some of our patients might be shorter than the observation period of 10 days, which could result in biases of clinical observation characteristics.

Conclusion

Compared with the symptoms of the initial patients with SARS-Cov-2 infection in Wuhan, those of patients from Zhejiang province in our study were relatively mild. Currently, no effective drug treatment or vaccine exists. It is necessary for monitoring of the virus to be strengthened and drugs and vaccines to be developed against SARS-Cov-2 infection as soon as possible.

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We thank Song-Jia Tang for assisting with English translation.

Contributors: X-WX and X-XW contributed equally to this article. XWX and LJL conceptualised the paper. XXW analysed the data, with input from JFS, KJX, XGJ, LJY, CLM, SBL, HYW, SZ, HNG, HLC, and YQQ. XWX and XXW wrote the initial draft with all authors providing critical feedback and edits to subsequent revisions. All authors approved the final draft of the manuscript. L-LJ is the guarantor. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

Funding: No funding.

Competing interests: All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Ethical approval: This study was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (2020IIT A0001).

Patient consent: Obtained.

Data sharing: No additional data available.

Transparency: The lead authors and manuscript's guarantor affirm that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

Dissemination to participants and related patient and public communities: No study participants were involved in the preparation of this article. The results of the article will be summarised in media

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press releases from the Zhejiang University and presented at relevant conferences.

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Supplementary information: Description of clinical centres and cases enrolled in this study, and case report form

BMJ: first published as 10.1136/bmj.m606 on 19 February 2020. Downloaded from http://www.bmj.com/ on 15 March 2023 by guest. Protected by copyright.

The Journal of Antibiotics
https://doi.org/10.1038/s41429-021-00430-5

FOI 26-3132 - Document 5

JARA Japan Antibiotics Research Association
The Society for Actinomycetes Japan

REVIEW ARTICLE

The mechanisms of action of Ivermectin against SARS-CoV-2: An evidence-based clinical review article

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Received: 11 May 2021 / Revised: 17 May 2021 / Accepted: 20 May 2021
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Abstract

Considering the urgency of the ongoing COVID-19 pandemic, detection of various new mutant strains and future potential re-emergence of novel coronaviruses, repurposing of approved drugs such as Ivermectin could be worthy of attention. This evidence-based review article aims to discuss the mechanism of action of ivermectin against SARS-CoV-2 and summarizing the available literature over the years. A schematic of the key cellular and biomolecular interactions between Ivermectin, host cell, and SARS-CoV-2 in COVID-19 pathogenesis and prevention of complications have been proposed.

Introduction

A relatively recent surge in zoonotic diseases has been noted over the past few decades. Several reasons could be responsible for this "spill-over" of disease-causing agents from animals to humans. These include an exponential rise in the global population causing man to encroach new ecological habitats in search of space, food, and resources as well as improved opportunities for rampant wildlife trade causing inter-species pathogen jumps. The 1980s was known for HIV/AIDS crisis that originated from the great apes, while the Avian flu pandemic in 2004-07 came from the birds. The pigs lead to the Swine flu pandemic in 2009 and bats were the original hosts of Ebola, Severe Acute Respiratory Syndrome (SARS), Middle Eastern respiratory syndrome (MERS), and probably Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) outbreak as well.

COVID-19 has already caused millions of deaths worldwide and has paralyzed not only the world's health-care system but also the political and economic relations between countries [1]. The fact that the SARS-CoV-2 virus has been thought to have originated from wildlife and may have "jumped" into humans, not only highlights future risks from animal-borne diseases but also provides an important clue to its resolution. In such a scenario, where this "jump" has been made from animal to human, it seems only logical to review a drug that has worked efficiently against a disease-causing agent and is available in a form that is safe for human consumption since the early 1980 s.

Ivermectin belongs to a group of avermectins (AVM), which is a group of 16 membered macrocyclic lactone compounds discovered at the Japanese Kitasato institute in 1967 during actinomycetes cultures with the fungus *Streptomyces avermitilis* [2]. This drug radically lowered the incidence of river blindness and lymphatic filariasis and was discovered and developed by William C. Campbell and Satoshi Ōmura for which they received the Nobel Prize in Physiology or Medicine in 2015 [3, 4]. Ivermectin is enlisted in the World Health Organization's Model List of Essential Medicines [5].

Drug repurposing, drug redirecting, or drug reprofiling is defined as the identification of novel usages for existing drugs. The development risks, costs as well as safety-related failure, are reduced with this approach since these drugs have a well-established formulation development, in vitro and in vivo screening, as well as pharmacokinetic and pharmacodynamic profiles. Moreover, the first clinical trial phases of many such drugs have been completed and can be bypassed to reduce several years of development. Therefore, drug repurposing has the potential to reduce the time frame for the whole process by up to 3–12 years and carries great potential [6].

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Published online: 15 June 2021

Table 1 All 55 ivermectin COVID-19 trials (As per data available on 16 May 2021) divided based on stage of treatment (Early Vs Late) and the type of study

Study	Study Type
EARLY TREATMENT	
Random effects meta-analysis with pooled effects showed 79% improvement for early treatment RR 0.21 and CI [0.11-0.37]	
Double-Blind Randomized controlled trial	Mahmud et al.*, Ahmed et al.*, Chaccour et al.*, Babalola et al.*, Kirti et al., Mohan et al., Schwartz et al., Lopez- Medina et al.*, Chahla et al.
Single-blind Randomized controlled trial	Raad et al.
Randomized controlled trial	Bukhari et al., Chowdhury et al.*, Faisal et al.*
Retrospective quasi-randomized study	Loue et al*, Merino et al
Other studies	Espitia-Hernandez et al.*, Carvallo et al., Cadegiani et al., Afsar et al., Elalfy et al.*, Roy et al., Mourya et al.*
LATE TREATMENT	
Random effects meta-analysis with pooled effects showed 46% improvement for late treatment RR 0.54 and CI [0.40-0.72]	
Randomized controlled trial	Kishoria et al.*, Podder et al.*, Chachar et al.*, Elgazzar et al.. Pott-Junior et al.*
Double-Blind Randomized controlled trial	Niaee et al.,Okumus et al.*, Shahbazn et al.*, Gonzalez et al.*. Huvemek et al.
Single-Blind Randomized controlled trial	Hashim et al.
Other studies	Gorial et al., Khan et al., Soto-Becerra et al, Rajter et al.*, Camprubi et al.*, Spoorthi et al*, Budhiraja et al., Lima Morales et al.*

The 29 peer-reviewed trials have been marked with an asterisk as a superscript. (*) (source: https://ivmmeta.com/)

Although several drugs received Emergency Use Authorization for COVID-19 treatment with unsatisfactory supportive data, Ivermectin, on the other hand, has been sidelined irrespective of sufficient convincing data supporting its use. Nevertheless, many countries adopted ivermectin as one of the first-line treatment options for COVID-19.

With the ongoing vaccine roll-out programs in full swing across the globe, the longevity of the immunity offered by these vaccines or their role in offering protection against new mutant strains is still a matter of debate. The adoption of Ivermectin as a "safety bridge" by some sections of the population that are still waiting for their turn for vaccination could be considered as a "logical" option.

Several doctor-initiated clinical trial protocols that aimed to evaluate outcomes, such as reduction in mortality figures, shortened length of intensive care unit stay and/or hospital stay and elimination of the virus with ivermectin use have been registered at the US ClinicalTrials.gov [7]. Real-time data is also available with a meta-analysis of 55 studies to date. As per data available on 16 May 2021, 100% of 36 early treatment and prophylaxis studies report positive effects (96% of all 55 studies). Of these, 26 studies show statistically significant improvements in isolation. Random effects meta-analysis with pooled effects using the most serious outcome reported 79% and 85% improvement for early treatment and prophylaxis respectively (RR 0.21 [0.11–0.37] and 0.15 [0.09–0.25]). The results were similar after exclusion based sensitivity analysis: 81% and 87% (RR 0.19 [0.14–0.26] and 0.13 [0.07–0.25]), and after restriction to 29 peer-reviewed studies: 82% and 88% (RR 0.18 [0.11–0.31] and 0.12 [0.05–0.30]). Statistically significant improvements were seen for mortality, ventilation, hospitalization, cases, and viral clearance. 100% of the 17 Randomized Controlled Trials (RCTs) for early treatment and prophylaxis report positive effects, with an estimated improvement of 73% and 83% respectively (RR 0.27 [0.18–0.41] and 0.17 [0.05–0.61]), and 93% of all 28 RCTs. These studies are tabulated in Table 1. The probability that an ineffective treatment generated results as positive for the 55 studies to date is estimated to be 1 in 23 trillion ($p = 0.000000000000043$). The consistency of positive results across a wide variety of cases has been remarkable. It is extremely unlikely that the observed results could have occurred by chance [8].

However, a controlled outpatient trial by López-Medina et al. demonstrated that, in mild COVID-19, Ivermectin showed no improvement [9]. Misinterpretation of results were noted due to possible gaps in regards to the study quality (study design, the methodology adopted, statistical analysis, and hence the conclusion).

Ivermectin has rapid oral absorption, high liposolubility, is widely distributed in the body, metabolized in the liver (cytochrome P450 system) and excreted almost exclusively in feces [4]. Following a standard oral dose in healthy humans, it reaches peak plasma levels at 3.4 to 5 h; and plasma half-life has been reported to be 12 to 66 h [10]. Despite its widespread use, there are relatively few studies

FOI 26-3132 - Document 5

Table 2 A list of studies demonstrating the role of Ivermectin (IVM) on SARS-CoV-2

MAIN ROLE OF IVERMECTIN AGAINST SARS-COV-2	STUDY AUTHORS	STUDY YEAR	REFERENCES
A. DIRECT ACTION ON SARS-COV-2			
Level 1: Action on SARS-CoV-2 cell entry			
IVM docks in the region of leucine 91 of the spike protein and histidine 378 of the ACE2 receptor	Leher et al.	2020	[22]
IVM has the highest binding affinity to the predicted active site of the S glycoprotein; Considerable binding affinity to the predicted active site of the SARS-CoV-2 RdRp protein; Highest binding affinity to the predicted active site of nsp14; highest binding affinity to the active site of the TMPRSS2 protein	Eweas et al.	2021	[23]
IVM utilizes viral spike protein, main protease, replicase, and human TMPRSS2 receptors as the most possible targets for executing its antiviral efficiency by disrupting binding	Choudhury et al.	2021	[24]
Level 2: Action on Importin (IMP) superfamily			
in presence of a viral infection, IVM targets the IMPα component of the IMP α/β1 heterodimer and binds to it, preventing interaction with IMP β1, subsequently blocking the nuclear transport of viral proteins.	Yang, S.N.Y et al.	2020	[26]
Level 3: Action as an Ionophore			
Two ivermectin molecules, reacting with each other in a "head-tail" mode, can create a complex suitable to be considered as ionophore. These ionophores allow neutralizing the virus at an early stage of the infection before it can adhere to the host cells and enter it.	Rizzo E et al.	2020	[28]
B. ACTION ON HOST TARGETS FOR VIRAL REPLICATION			
Level 4: Action as an antiviral			
IVM has antiviral properties against other viruses including the RNA viruses such as Zika Virus (ZKV), Dengue virus, yellow fever virus (YFV), and West Nile virus (WNV), Hendra virus (HEV), Newcastle virus, Venezuelan equine encephalitis virus (VEEV), Chikungunya virus (CHIKV), Semliki Forest virus (SFV), and Sindbis virus (SINV), Avian influenza A virus, Porcine Reproductive and Respiratory Syndrome virus (PRRSV), Human immunodeficiency virus type 1 as well as DNA viruses such as Equine herpesvirus type 1 (EHV-1) and Pseudorabies virus (PRV).	Heidary, F et al.	2020	[29]
Level 5: Action on viral replication and assembly			
In Vero/hSLAM cells infected with the SARS-CoV-2 virus when "exposed" to 5 μM IVM showed a 5000-fold reduction in viral RNA at 48 h when compared to the control group	Caly L et al.	2020	[30]
utilizing modeling approach, predicted lung accumulation of Ivermectin over 10 times higher than EC 50	Arshad et al	2020	[31]
best binding interaction between IVM and RNA-dependent RNA polymerase (RdRp)	Swargiary et al.*	2020	[33]
highly efficient binding of IVM to nsp14	Ma et al.	2015	[35]
highly efficient binding of IVM to the viral N phosphoprotein and M protein	Eweas et al.	2021	[23]
Level 6: Action on post-translational processing of viral polyproteins			
IVM binds to both proteins, Mpro, and to a lesser extent to PLpro of SARS-CoV-2	Eweas et al.	2021	[23]
Level 7: Action on Karyopherin (KPNA/KPNB) receptors			
IVM inhibits the KPNA/KPNB1- mediated nuclear import of viral proteins	Caly L et al.	2020	[30]
C. ACTION ON HOST TARGETS FOR INFLAMMATION			
Level 8: Action on Interferon (INF) levels			
IVM promotes the expression of several IFN-related genes, such as IFIT1, IFIT2, IF144, ISG20, IRF9, and OASL	Seth C	2016	[40]
Level 9: Action on Toll- like-Receptors (TLRs)			
IVM blocks activation of NF-kappa B pathway and inhibition of toll-like receptor 4 (TLR4) signaling	Zhang X et al.	2008	[42]
Level 10: Action on Nuclear Factor-κB (NF-κB) pathway			
IVM at its very low dose, which did not induce cytotoxicity, drastically reversed the resistance of tumor cells to the chemotherapeutic drugs both in vitro and in vivo by inhibition of the transcriptional factor NF-κB.	Jiang L et al.	2019	[44]

Table 2 (continued)

MAIN ROLE OF IVERMECTIN AGAINST SARS-COV-2	STUDY AUTHORS	STUDY YEAR	REFERENCES
IVM inhibits lipopolysaccharide (LPS)-induced production of inflammatory cytokines by blocking the NF-κB pathway and improving LPS-induced survival in mice.	Zhang X et al.	2008	[42]
Level 11: Action on the JAK-STAT pathway, PAI-1 and COVID-19 sequalae			
IVM inhibits STAT-3, SARS-CoV-2-mediated inhibition of IFN and STAT 1, with the subsequent shift to a STAT 3- dominant signaling network that could result in almost all of the clinical features of COVID-19; STAT-3 acts as a "central hub" that mediates the detrimental COVID-19 cascade	Matsuyama, T.,	2020	[39]
STAT-3 induces a C-reactive protein that upregulates PAI-1 levels. Ivermectin inhibits STAT-3.	Matsuyama, T.,	2020	[39]
The PD-L1 receptors present on the endothelial cells are activated by STAT-3 causing T cell lymphopenia. IVM inhibits STAT-3 through direct inhibition	Matsuyama, T.,	2020	[39]
Level 12: Action on P21 activated Kinase 1 (PAK-1)			
IVM suppresses the Akt/mTOR signaling and promotes ubiquitin-mediated degradation of PAK-1 hence compromising STAT-3 activity and decreasing IL-6 production.	Dou Q et al.	2016	[54]
Level 13: Action on Interleukin-6 (IL-6) levels			
IVM suppressed IL-6 and TNFα production	Zhang X et al.	2008	[42]
IVM "dramatically reduced" IL-6/IL-10 ratio modulating infection outcomes.	G D de Melo et al. *	2020	[55]
Level 14: Action on allosteric modulation of P2X4 receptor			
Positive allosteric modulation of $P2X_4$ by IVM enhances ATP-mediated secretion of CXCL5	Layhadi JA et al.	2018	[58]
Level 15: Action on high mobility group box 1 (HMGB1)			
Ivermectin inhibits HMGB1	Juarez M et al.	2018	[60]
Level 16: Action as an immunomodulator on Lung tissue and olfaction			
No olfactory deficit was observed in IVM-treated females; IVM dramatically reduced the IL-6/IL-10 ratio in lung	G D de Melo et al. *	2020	[55]
Level 17: Action as an anti-inflammatory			
anti-inflammatory action of IVM was explained as inhibition of cytokine production by lipopolysaccharide challenged macrophages, blockade of activation of NF-kB, and the stress-activated MAP kinases JNK and p38, and inhibition of TLR4 signaling.	Zhang X et al., Ci X et al., Yan S et al.	2008 2009 2011	[42, 62, 63]
Immune cell recruitment, cytokine production in bronchoalveolar lavage fluid, IgE, and IgG1 secretion in serum as well as hyper-secretion of mucus by goblet cells was reduced significantly by IVM	Yan S et al.	2011	[63]
D. ACTION ON OTHER HOST TARGETS			
Level 18: Action on Plasmin and Annexin A2			
Annexin acts as a co-receptor for the conversion of plasminogen to plasmin in the presence of t-PA. increased levels of plasmin leads to direct activation of STAT-3.	Kamber Zaidi et al.	2020	[64]
IVM directly inhibits STAT-3 and could play a role in the inhibition of COVID-19 complications.	Matsuyama et al.	2020	[39]
Level 19: Action on CD147 on the RBC			
The SARS-CoV-2 does not internalize into the red blood cells but such attachments can lead to clumping. IVM binds to the S protein of the SARS-CoV-2 virus making it unavailable to bind with CD147.	David E.Scheim et al.	2020	[65]
Level 20: Action on mitochondrial ATP under hypoxia on cardiac function			
IVM increased mitochondrial ATP production by inducing Cox6a2 expression and maintains mitochondrial ATP under hypoxic conditions. This prevents pathological hypertrophy and improves cardiac function.	Nagai H et al.	2017	[67]

*available as preprint; Clinical trials of IVM on COVID-19 available on https://clinicaltrials.gov[7]; Ivermectin for COVID-19: real-time meta-analysis available on https://ivmmeta.com [8]

FOI 26-3132 - Document 5

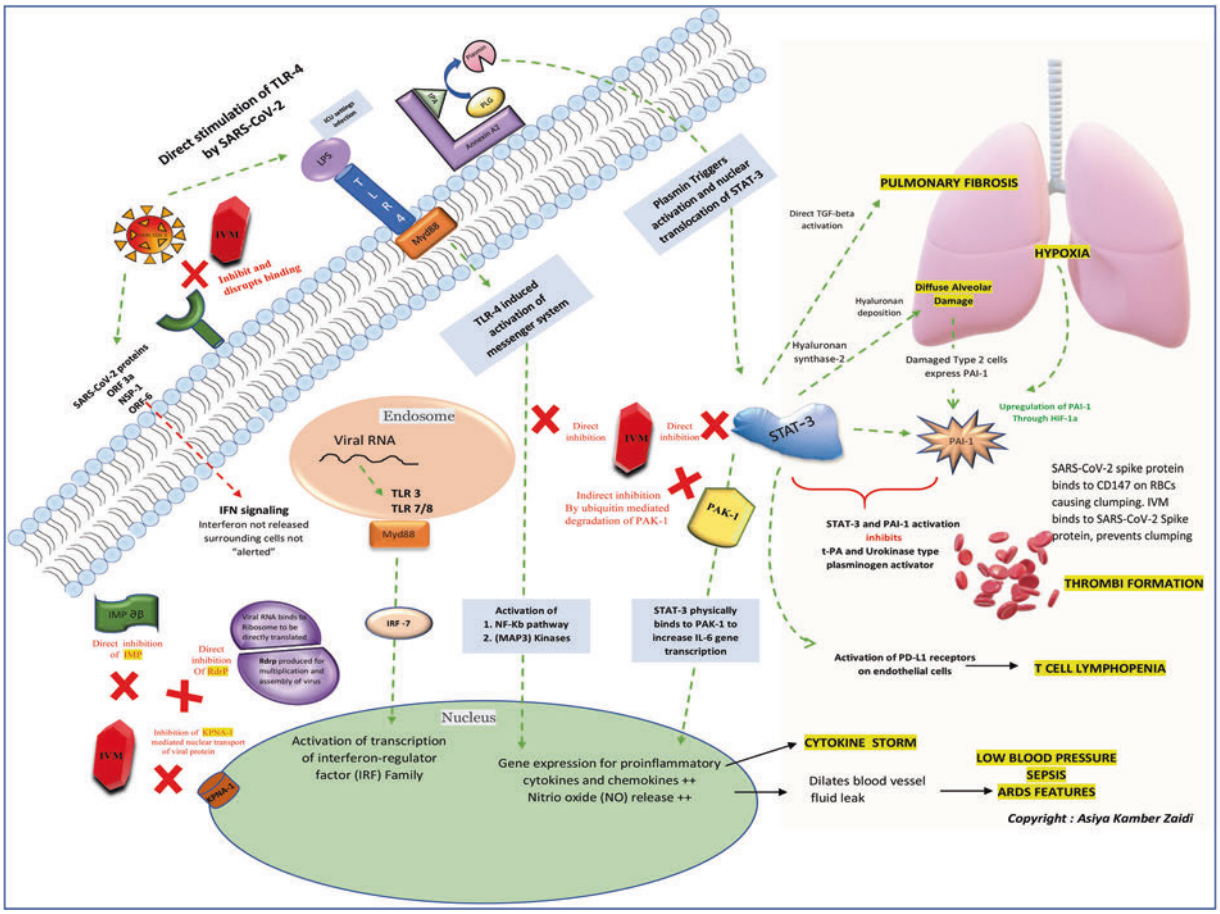


Fig. 1 A schematic of the key cellular and biomolecular interactions between Ivermectin, host cell, and SARS-CoV-2 in COVID-19 pathogenesis and prevention of complications. Ivermectin; IVM (red block) inhibits and disrupts binding of the SARS-CoV-2 S protein at the ACE-2 receptors (green). The green dotted lines depict activation pathways and the red dotted lines depict the inhibition pathways. The TLR-4 receptors are directly activated by SARS-CoV-2 and also by LPS mediated activation (seen during ICU settings) causing activation of NF-Kb pathway and MAP3 Kinases leading to increased intranuclear gene expression for proinflammatory cytokines and chemokines (responsible for cytokine storm) and NO release (responsible for blood vessel dilatation, fluid leak, low blood pressure, ARDS and sepsis). The NF-Kb and STAT-3 pathway activation is central to the pathogenesis and sequelae of COVID-19. STAT-3 physically binds to PAK-1 and increases IL-6 transcription. The annexin A2 at the cell surface converts plasminogen; PLG to plasmin under the presence of t-PA. Plasmin triggers activation and nuclear translocation of STAT-3. An upregulation of STAT-3 stimulates hyaluronan synthase-2 in the lung cells causing hyaluronan deposition leading to diffuse alveolar damage and hypoxia. STAT-3 also directly activates TGF-beta initiating pulmonary fibrosis; a typical characteristic of SARS-COV-2 lung pathology. The damaged type 2 cells express PAI-1 and an already hypoxic state also causes an upregulation of PAI (through Hypoxic inducible factor-1) along with direct stimulation by STAT-3. Simultaneous STAT-3 and PAI-1 activation inhibits t-PA and urokinase-type plasminogen activator leading to thrombi formation. Also, the SARS-CoV-2 spike protein binds to the CD147 on red blood cells and causes clumping. *IVM in turn, binds to SARS-CoV-2 Spike protein and hence prevents clumping*. T cell lymphopenia in COVID-19 can also be attributed to the direct activation of PD-L1 receptors on endothelial cells by STAT-3. *IVM directly inhibits the NF-kb pathway, STAT-3, and indirectly inhibits PAK-1 by increasing its ubiquitin-mediated degradation*. The natural antiviral response of a cell is through interferon regulatory genes and viral RNA mediated activation of TLR-3 and TLR7/8- Myd88 activation of transcription of interferon-regulator (IRF) family. For a virus to establish an infection, this antiviral response needs to be inhibited by blocking interferon production. The proteins such as importin and KPNA mediate nuclear transport of viral protein and subsequent IFN signaling. The SARS-CoV-2 proteins (ORF-3a, NSP-1, and ORF-6) directly block IFN signaling causing the surrounding cells to become unsuspecting victims of the infection. *IVM inhibits both importin a-b (green) as well as the KPNA-1 receptors (brown) causing natural antiviral IFN release. IVM also inhibits viral RdrP, responsible for viral replication*. *IVM* Ivermectin, *ACE-2* angiotensin-converting-enzyme 2, *LPS* Lipopolysaccharide, *TLR* Toll-like receptor, *t-PA* tissue-like plasminogen activator, *PLG* Plasminogen, *IMPab* Importin alpha-beta, *Rdrp* RNA dependant RNA polymerase, *KPNA-1* Karyopherin Subunit Alpha 1, *NF-kB* nuclear factor kappa-light-chain-enhancer of activated B cells, *Map3Kinases* Mitogen-activated Kinases, *PAK-1* P21 Activated Kinase 1, *STAT-3* Signal transducer and activator of transcription 3, *PAI-1* Plasminogen activator inhibitor-1, *HIF-1* Hypoxia-Inducible Factor

on the pharmacokinetics of Ivermectin in humans [11]. Ivermectin binds strongly to plasma proteins in healthy subjects (93.2%) [12]. Such an "avid binding" can be beneficial when administered in countries where malnutrition and hypoalbuminemia are common, leading to an increased availability of "free fraction" of ivermectin [4].

Hypoalbuminemia is a frequent finding in patients with COVID-19 and it also appears to be linked to the severity of lung injury [13]. Therefore, Ivermectin might be useful when used in such a setting.

There is evidence supporting the use of Ivermectin in decreasing mortality figures in patients with SARS-CoV-2 infection. However, the use of ivermectin orally in an outpatient setting also requires strict and well defined guidelines to avoid any form of overdosing that could lead to toxicity. A study by Baudou, E et. al described two human ABCB1 nonsense mutations associated with a loss of function in a patient who had an adverse reaction to ivermectin after the administration of a usual dose. This finding warrants caution regarding medical prescriptions of ivermectin and other ABCB1 substrates [14].

This article aims to discuss the mechanism of action by summarizing the in vitro and in vivo evidence demonstrating the role of Ivermectin in COVID-19 as per the available literature over the years. [Table 2] A schematic of the key cellular and biomolecular interactions between Ivermectin, host cell, and SARS-CoV-2 in COVID-19 pathogenesis and prevention of complications has been proposed. [Fig. 1]

Methods

A comprehensive search of the PubMed database was conducted from January 1, 2008 up to January 30, 2021 using syntax constructed using MeSH Database as follows: (stromectol OR Ivermectin OR "dihydroavermectin") OR (22 AND 23-dihydroavermectin B) AND (antiviral OR virus OR COVID-19 OR SARS-CoV-2). All the results obtained were manually reviewed for content, relevance and included when considered appropriate. The papers cited in the references were also reviewed and included when considered appropriate. The articles were retrieved manually to exclude any duplicates.

Results

Ivermectin as an anti-helminth

Ivermectin has been approved as an anti-helminthic [15]. It is a selective positive allosteric modulator at the glutamate-gated chloride channels found in nematodes and insects and acts by binding to these channels leading to chloride ion influx causing hyperpolarization of the cell and hence, dysfunction [16]. However, at higher concentrations, Ivermectin can also bind to host GABA receptors only when the blood-brain barrier (BBB) is "leaky". This is not the case in healthy human beings with an intact BBB as the drug is "excluded" by a *p*-glycoprotein drug pump (MDR-1). Chandler et al. considered Ivermectin to be free of potential neurological adverse drug reactions, except in situations of overdose [17].

SARS-CoV-2 virus structure

SARS-CoV-2 is a sarbecovirus with structural similarity to SARS-CoV-1. Out of the four structural proteins of the SARS-CoV-2 beta coronavirus, namely: Spike (S) protein, membrane (M) protein, envelope (E) protein, and nucleocapsid (N) protein, the S protein is responsible for eliciting potent neutralizing antibody responses. The entry of SARS-CoV-2 into the host cell is mediated by the binding of the S1 subunit of its S protein (receptor binding domain) to the Angiotensin-converting enzyme 2 (ACE-2) receptors present on the host cell surface [18]. The S2 subunit is associated with a fusion protein that binds with the cell membrane after priming with Transmembrane protease, serine 2 (TMPRSS-2) and is responsible for fusion with the host cell.

The SARS-CoV-2 genome consists of ~29.8 kb nucleotides; it possesses 14 open reading frames (ORFs) encoding 27 proteins [19]. The 5′ two-thirds of the viral genome encodes the replicase gene. It contains two ORFs: ORF1a and ORF1b. ORF1a/b encodes two polyproteins by polymerase frameshifting; these are then post-translationally cleaved into 15 non-structural proteins (nsps): nsp1–10 and nsp12–16. The rest of the genome encodes for the four structural proteins [(S protein, E protein, M protein, N protein], in addition to eight accessory proteins (3a/3b, p6, 7a/7b, 8b, 9b, and ORF14) [19]. The replicase also encodes the papain-like protease (PLpro) and the serine-type protease or main protease (Mpro) [20].

In principle, a molecule can act as an anti-viral drug if it "inhibits some stage of the virus replication cycle, without being too toxic to the body's cells [21]."

The possible modes of action of anti-viral agents would include the following:

1. Inactivate extracellular virus particles.
2. Prevent viral attachment and/or entry.
3. Prevent replication of the viral genome.
4. Prevent synthesis of specific viral protein(s).
5. Prevent assembly or release of new infectious virions

The role of Ivermectin against the SARS-CoV-2 virus

The targets of activity of Ivermectin can be divided into the following four groups:

A. *Direct action on SARS-CoV-2*
   Level 1: Action on SARS-CoV-2 cell entry
   Level 2: Action on Importin (IMP) superfamily
   Level 3: Action as an Ionophore
B. *Action on host targets important for viral replication*
   Level 4: Action as an antiviral
   Level 5: Action on viral replication and assembly
   Level 6: Action on post-translational processing of viral polyproteins
   Level 7: Action on Karyopherin (KPNA/KPNB) receptors
C. *Action on host targets important for inflammation*
   Level 8: Action on Interferon (INF) levels
   Level 9: Action on Toll- like-Receptors (TLRs)
   Level 10: Action on Nuclear Factor-κB (NF-κB) pathway
   Level 11: Action on the JAK-STAT pathway, PAI-1 and COVID-19 sequalae
   Level 12: Action on P21 activated Kinase 1 (PAK-1)
   Level 13: Action on Interleukin-6 (IL-6) levels
   Level 14: Action on allosteric modulation of P2X4 receptor
   Level 15: Action on high mobility group box 1 (HMGB1),
   Level 16: Action as an immunomodulator on Lung tissue and olfaction
   Level 17: Action as an anti-inflammatory
D. *Action on other host targets*
   Level 18: Action on Plasmin and Annexin A2
   Level 19: Action on CD147 on the RBC
   Level 20: Action on mitochondrial ATP under hypoxia on cardiac function

The direct "antiviral targets" may be useful in the early stages while the anti-inflammatory targets might be addressed in the later stages of the disease.

Direct action of Ivermectin on SARS-CoV-2

Level 1: Action on SARS-CoV-2 cell entry

A study by Lehrer S et al observed that Ivermectin docked in the region of leucine 91 of the SARS-CoV-2 spike protein and histidine 378 of the host cell ACE-2 receptor blocking its entry into the host cell [22]. In yet another study by Eweas et al., potential repurposed drugs such as Ivermectin, chloroquine, hydroxychloroquine, remdesivir, and favipiravir were screened and molecular docking with different SARS-CoV-2 target proteins including S and M proteins, RNA-dependent RNA polymerase (RdRp), nucleoproteins, viral proteases, and nsp14, was performed. Ivermectin showed the following 5 important docking properties [23]:

1. Highest binding affinity to the predicted active site of the S glycoprotein (Mol Dock score −140.584) and protein–ligand interactions (MolDock score−139.371).
2. Considerable binding affinity to the predicted active site of the SARS-CoV-2 RdRp protein (MolDock score −149.9900) and protein–ligand interactions (MolDock score −147.608), it formed H-bonds with only two amino acids: Cys622 and Asp760.
3. Highest binding affinity (MolDock score −212.265) to the predicted active site of nsp14.
4. The highest binding affinity to the active site of the TMPRSS2 protein (MolDock score −174.971) and protein–ligand interactions (MolDock score −180.548). Moreover, it formed five H-bonds with Cys297, Glu299, Gln438, Gly462, and Gly464 amino acid residues present at the predicted active site of the TMPRSS protein
5. The free binding energy of the spike protein (open) was higher in Ivermectin (−398.536 kJ/mol) than remdesivir (−232.973 kJ/mol).

An In-silico data analysis conducted by Choudhury et al. demonstrated that Ivermectin efficiently utilizes viral spike protein, main protease, replicase, and human TMPRSS2 receptors as the most possible targets for executing its "antiviral efficiency" by disrupting binding. Since Ivermectin exploits protein targets from both, the virus and human, this could be the behind its excellent in vitro efficacy against SARS-CoV-2 [24].

The development of vaccines for SARS-CoV-2 is centered around spike protein biology (virus targeted) and the recently documented "vaccine escape strains" have been a cause of worry. In such a situation, Ivermectin, is both, virus as well as host targeted and hence could act as a potential therapeutic against these new strains that could "escape" immunity offered by the vaccine.

Level 2: Action on Importin (IMP) superfamily

Inside the cell, the nuclear transport of proteins into and out of the nucleus is signal-dependent and mediated by the Importin (IMP) superfamily of proteins that exist in α and β forms. This IMPα/β1 exists as a heterodimer with a "IBB" (IMP β-binding) site present over IMP α that binds to IMP β1 on "cargo recognition" by IMPα. The SARS-CoV-2 virus upon host cell entry tends to "load" its proteins over the host protein IMP α/β1 heterodimer (importin) to enter the nucleus through the nuclear pore complex. Once inside, the importin molecule detaches while the viral protein from the SARS-CoV-2 virus hijacks the host cell machinery and inhibits the natural cell "anti-viral" response by blocking the release of

interferon (an antiviral substance released by an infected cell to alert the surrounding cells of an ongoing viral attack). As a result, the surrounding cells become "unsuspecting victims" of the virus and the infection continues with the virus escaping recognition by the immune cells [25]. Ivermectin, in presence of a viral infection, targets the IMPα component of the IMP α/β1 heterodimer and binds to it, preventing interaction with IMP β1, subsequently blocking the nuclear transport of viral proteins. This allows the cell to carry out its normal antiviral response [26]. In such a case, it should be noted that the activity of Ivermectin here is viro-static, that is, it neutralizes the virus by competing for the same receptor.

Level 3: Action as an Ionophore

Ionophores are molecules that typically have a hydrophilic pocket which constitutes a specific binding site for one or more ions (usually cations), while its external surface is hydrophobic, allowing the complex thus formed to cross the cell membranes, affecting the hydro-electrolyte balance [27]. It can be hypothesized that two ivermectin molecules, reacting with each other in a "head-tail" mode, can create a complex suitable to be considered such [28]. These ionophores allow neutralizing the virus at an early stage of the infection before it can adhere to the host cells and enter it to exploit their biochemical machinery for the production of other viral particles.

Action on host targets for viral replication

Level 4: Action as an antiviral

A systematic review article by Heidary, F. discussed the "anti-viral" properties of Ivermectin against other viruses including the RNA viruses such as Zika Virus (ZKV), Dengue virus, yellow fever virus (YFV), and West Nile virus (WNV), Hendra virus (HEV), Newcastle virus, Venezuelan equine encephalitis virus (VEEV), Chikungunya virus (CHIKV), Semliki Forest virus (SFV), and Sindbis virus (SINV), Avian influenza A virus, Porcine Reproductive and Respiratory Syndrome virus (PRRSV), Human immunodeficiency virus type 1 as well as DNA viruses such as Equine herpesvirus type 1 (EHV-1) and Pseudorabies virus (PRV) [29].

Level 5: Action on viral replication and assembly

An in-vitro study by Caly L et al. demonstrated that the Vero/hSLAM cells infected with the SARS-CoV-2 virus when "exposed" to 5 μM Ivermectin showed a 5000-fold reduction in viral RNA at 48 h when compared to the control group [30]. This study attracted opinions regarding the inability of Ivermectin to achieve the therapeutic effect of COVID-19 through routine dosage. Contrary to this, Arshad et al, by utilizing modeling approach, predicted lung accumulation of Ivermectin over 10 times higher than EC_{50}. This likelihood of attainment of higher lung tissue concentrations of Ivermectin leaves the door open for further research especially for respiratory infections [31].

An explanation for the study by Caly et al was provided in a review article: Global trends in clinical studies of ivermectin in COVID-19 by Yagisawa et al., co-authored by Prof. Satoshi Ōmura, regarding the "setting of the sensitivity for experimental systems in vitro". As per the authors, using Vero/hSLAM cells, the antiviral activity of the test drug was reliably measured and the sensitivity of the $IC_{50} = 2\,\mu M$ set by them was appropriate as neither false positives nor false negatives occurred. Therefore, the study by Caly et al. merely indicated that ivermectin was found to have anti-SARS-CoV-2 activity in vitro—no more, no less. Also, the fact that there are in vivo infection experiments that could be used to connect in vitro experiments to clinical studies [32].

Another in-silico study by Swargiary et al. demonstrated the best binding interaction of −9.7 kcal/mol between Ivermectin and RdRp suggesting inhibition of viral replication [33]. The RdRP residing in nsp12 is the centerpiece of the coronavirus replication and transcription complex and has been suggested as a promising drug target as it is a crucial enzyme in the virus life cycle both for replication of the viral genome but also for transcription of subgenomic mRNAs (sgRNAs) [34]. Ivermectin binds to the viral rdrp and disrupts it. The highly efficient binding of ivermectin to nsp14 confirms its role in inhibiting viral replication and assembly. It is well known that nsp14 is essential in transcription and replication. It acts as a proofreading exoribonuclease and plays a role in viral RNA capping by its methyltransferase activity [35]. Moreover, highly efficient binding of ivermectin to the viral N phosphoprotein and M protein is suggestive of its role in inhibiting viral replication and assembly [23].

Level 6: Action on post-translational processing of viral polyproteins

Once gaining entry into the host cell, the viral RNA is translated by the host ribosome into a large "polyprotein". Some enzymes break away through autoproteolysis from this polyprotein and further help other proteins to break off and carry out their function for replication. One such enzyme, 3 chymotrypsin-like proteases (3'cl pro/ Mpro) is responsible for working on this polyprotein causing other proteins to "librate" and carry out viral replication. Ivermectin binds to this enzyme and disrupts it. It also efficiently binds to both proteins, Mpro, and to a lesser extent to PLpro of SARS-CoV-2; therefore, it has a role in preventing the post-translational processing of viral polyproteins [23].

Level 7: Action on Karyopherin (KPNA/KPNB) receptors

Karyopherin-α1 (KPNA1) is essential for the nuclear transport of signal transducers and activators of transcription 1 (STAT1) [36], and the interaction between STAT1 and KPNA1 (STAT1/KPNA1) involves a nonclassical nuclear

localization signal (NLS). Ivermectin inhibits the KPNA/KPNB1- mediated nuclear import of viral proteins allowing the cell to carry out its normal antiviral response [30].

Action on host targets for inflammation

Level 8: Action on Interferon (INF) levels

These virus-infected cells release interferons that bind to the IFN receptors present on neighboring cells alerting them of a viral attack. The IFN-I and IFN-III receptors then further activate members of the JAK-STAT family. The virus after gaining entry into the host cell hijacks the host cell machinery and works towards antagonizing the normal interferon-mediated host cell antiviral response. SARS-CoV-2 proteins such as ORF3a, NSP1, and ORF6 inhibit IFN-I signaling [37, 38]. As a result, the cells surrounding the SARS-CoV-2 virus-infected cell "fail" to receive "critical and protective IFN signals" causing this SARS-CoV-2 virus to replicate and spread without any hindrance. This is one of the main reasons that, at this stage, COVID-19 infection is "hard to detect" clinically [39].

Ivermectin has been shown to promote the expression of several IFN-related genes, such as IFIT1, IFIT2, IF144, ISG20, IRF9, and OASL [40].

Level 9: Action on Toll- like-Receptors (TLRs)

Upon virus entry, the intracellular pattern recognition receptors (PRRs) present on the host cells are responsible for detecting the viral attack. The virus activates one such PRR named the Toll-like receptors (TLRs). These receptors are present on various immune system cells that help them locate and bind with the pathogen. The activation of TLRs, causes oligomerization, further activating downstream interferon regulatory factors (IRFs) and nuclear factor-kappa B (NF-kB) transcription factors inducing INF production [41]. Ivermectin plays a role in the blockade of activation of NF-kB pathway and inhibition of TLR4 signaling [42].

Level 10: Action on Nuclear Factor-κB (NF-κB) pathway

Activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines [43]. Jiang et al. demonstrated that Ivermectin at its very low dose, which did not induce cytotoxicity, drastically reversed the resistance of tumor cells to the chemotherapeutic drugs both in vitro and in vivo by inhibition of the transcriptional factor NF-κB [44]. Also, Zhang et al., suggested that Ivermectin inhibits lipopolysaccharide (LPS)-induced production of inflammatory cytokines by blocking the NF-κB pathway and improving LPS-induced survival in mice [42]. Therefore, using Ivermectin would be helpful in ICU settings where there are increased chances of bacterial infections (LPS mediated).

Level 11: Action on the JAK-STAT pathway, PAI-1 and COVID-19 sequalae

A strong correlation exists between SARS-CoV-2 viral load, disease severity, and progression [45]. COVID-19 not only causes flu-like symptoms such as fever, dry cough but could also lead to widespread thrombosis with micro-angiopathy in pulmonary vessels [46], raise D-dimer levels [47], cause lymphopenia [48], raise proinflammatory cytokine and chemokine production [49] as well as lead to a significant elevation of CRP levels [50]. SARS-CoV-2 has structural similarity with SARS-CoV-1. Several SARS-CoV-1 proteins antagonize the antiviral activities of IFNs and the downstream JAK (Janus kinase)-STAT signaling pathways they activate. JAK family kinases display a wide range of functions in ontogeny, immunity, chronic inflammation, fibrosis, and cancer [51].

The host proteins, such as the members of the signal transducers and activators of transcription (STATs) and NF-κB, enter the nucleus through nuclear envelope-embedded nuclear pores mediated by the IMPα/β1 heterodimer and play a role in COVID-19 pathogenesis. Frieman et al. demonstrated that accessory SARS ORF6 antagonizes STAT1 function by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane [52]. A review article by Matsuyama et al, hinted at SARS-CoV-2-mediated inhibition of IFN and STAT 1, with the subsequent shift to a STAT 3 dominant signaling network that could result in almost all of the clinical features of COVID-19 [39].

Before discussing further, it is important to understand the link between STAT-3 upregulation and COVID-19 sequelae and the role of Ivermectin in inhibiting STAT-3. STAT-3 acts as a "central hub" that mediates the detrimental COVID-19 cascade. In the lungs, STAT-3 activates Hyaluronan synthase-2 leading to deposition of hyaluronan causing diffuse alveolar damage. The damaged type 2 alveolar cells express PAI-1 (plasminogen activator inhibitor-1). Additionally, hypoxia due to diffuse alveolar damage causes an upregulation of PAI-1 through HIF-1a. STAT-3 also directly activates PAI-1. The simultaneous activation of PAI-1 and STAT-3 inhibits t-PA and urokinase-type plasminogen activator leading to thrombi formation in the capillaries. PAI-1 also binds to TLR-4 receptors on macrophages further activating the NF-kB pathway.

The "cytokine storm" typical of severe COVID-19 involves STAT-3 mediated upregulation of proinflammatory cytokines, TNFα, and IL-6 in macrophages. Additionally, STAT-3 induces a C-reactive protein that upregulates PAI-1 levels. STAT-3 is directly responsible for activating IL-6 gene transcription which further leads to an increase in TGF-β causing pulmonary fibrosis. The PD-L1 receptors present on the endothelial cells are activated by

STAT-3 causing T cell lymphopenia. Ivermectin inhibits STAT-3 through direct inhibition preventing COVID-19 sequalae [39].

Level 12: Action on P21 activated Kinase 1 (PAK-1)

The p21 activated kinase 1 (PAK1) physically binds to both JAK1 and STAT3, and the resultant PAK1/STAT3 complex activates IL-6 gene transcription responsible for cytokine storm in COVID-19 [53]. Ivermectin suppresses the Akt/mTOR signaling and promotes ubiquitin-mediated degradation of PAK-1 hence compromising STAT-3 activity and decreasing IL-6 production [54].

Level 13: Action on Interleukin-6 (IL-6) levels

A study by Zhang et al. demonstrated that Ivermectin suppressed IL-6 and TNFα production, two major components of the detrimental cytokine storm induced by SARS-CoV-2 and "dramatically reduced" IL-6/IL-10 ratio modulating infection outcomes [42, 55].

Level 14: Action on allosteric modulation of P2X4 receptor

P2X receptors are the channels selective to cation, are gated by extracellular ATP [56] and mediate several functions in health and disease [57]. From the seven subunits of P2X receptors, $P2X_4$ is most sensitive to Ivermectin. Positive allosteric modulation of $P2X_4$ by Ivermectin enhances ATP-mediated secretion of CXCL5 (pro-inflammatory chemokine). CXCL5 is a chemo-attractant molecule expressed in inflammatory cells in different tissues and modulates neutrophil chemotaxis and chemokine scavenging [58].

Level 15: Action on high mobility group box 1 (HMGB1)

The damage-associated molecular pattern high mobility group box 1 (HMGB1), is released by damaged cells acting as an agonist for the TLR4 receptor and hence mediating lung inflammation associated with COVID-19 [59]. Ivermectin inhibits HMGB1 [60].

Level 16: Action as an immunomodulator on Lung tissue and olfaction

In a study by DeMelo et al., the effects of Ivermectin were investigated on SARS-CoV-2 infection using the golden Syrian hamster as a model for COVID-19. Both, male and female adult golden Syrian hamsters were intranasally inoculated with 6×10^4 PFU of SARS-CoV-2. At the time of infection, animals received a single subcutaneous injection of Ivermectin (antiparasitic dose of 400 µg/kg) classically used in a clinical setting and were monitored over four days. Mock-infected animals received the physiological solution only. Interestingly, Ivermectin had a sex-dependent and compartmentalized immunomodulatory effect, preventing clinical deterioration and reducing the olfactory deficit in infected animals. This effect was sex-dependent: infected males presented a reduction in the clinical score whereas a complete absence of signs was noticed in the infected females. Regarding the olfactory performance, 83.3% (10/12) of the saline-treated males presented with hyposmia/anosmia, in contrast to only 33.3% (4/12) of IVM-treated males (Fisher's exact test $p = 0.036$). No olfactory deficit was observed in IVM-treated females (0/6), while 33.3% (2/6) of saline-treated females presented with hyposmia/anosmia (Fisher's exact test $p = 0.455$). Ivermectin dramatically reduced the IL-6/IL-10 ratio in lung tissue, which likely accounts for the more favorable clinical presentation in treated animals [55]. Loss of smell has been reported as one of the common symptoms in COVID-19 [61]. Interestingly, majority of patients in India regained their sense of smell after a brief anosmic period during their clinical course. Ivermectin is being used in India as one of the first-line drugs for COVID-19 treatment. It could be hypothesized that Ivermectin might have a role to play in reducing SARS-CoV-2 induced olfactory deficit.

Level 17: Action as an anti-inflammatory

The mechanism for anti-inflammatory action of Ivermectin was explained as inhibition of cytokine production by lipopolysaccharide challenged macrophages, blockade of activation of NF-kB, and the stress-activated MAP kinases JNK and p38, and inhibition of TLR4 signaling [42, 61, 62]. Moreover, Immune cell recruitment, cytokine production in bronchoalveolar lavage fluid, IgE, and IgG1 secretion in serum as well as hyper-secretion of mucus by goblet cells was reduced significantly by Ivermectin [63].

Action on other host targets

Level 18: Action on Plasmin and Annexin A2

As per study by Kamber Zaidi et al, annexin A2 may be linked to COVID-19 pathophysiology. Annexin A2 acts as a co-receptor for the conversion of plasminogen to plasmin in the presence of t-PA. Increased plasmin levels are found in co-morbid states and is also responsible for early stages of viral infection. Plasmin leads to direct activation of STAT-3 inducing detrimental COVID-19 sequelae. Ivermectin directly inhibits STAT-3 and could play a role in the inhibition of COVID-19 complications.

Level 19: Action on CD147 on the RBC

The transmembrane receptor CD147, present on the red blood cell (RBC) along with ACE-2 has been recognized as a key binding site for SARS-CoV-2 spike protein. The SARS-CoV-2 does not internalize into the RBC but such attachments can lead to clumping [65]. Ivermectin binds to the S protein of the virus making it unavailable to bind with CD147. This action might also be beneficial in advanced stages of COVID-19 presenting with clotting/thrombotic phenomena.

Level 20: Action on mitochondrial ATP under hypoxia on cardiac function

SARS-CoV-2 has been a well-known cause for acute myocardial injury and chronic damage to the cardiovascular

FOI 26-3132 - Document 5

system in active infection as well as in long haulers [66]. Nagai et al. demonstrated that Ivermectin increased mitochondrial ATP production by inducing Cox6a2 expression and maintains mitochondrial ATP under hypoxic conditions preventing pathological hypertrophy and improving cardiac function [67].

Conclusion

Considering the urgency of the ongoing COVID-19 pandemic, simultaneous detection of various new mutant strains and future potential re-emergence of novel coronaviruses, repurposing of approved drugs such as Ivermectin could be worthy of attention.

Acknowledgements We would like to thank all the academicians, physicians, and scientists dedicating their efforts and time to COVID-19 research. A special thank you to children who bring positivity and hope in these difficult times, especially Ginevra Dehgani.

Compliance with ethical standards

Conflict of interest The authors declare no competing interest.

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FOI 26-3132 - Document 5
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Inflamm. res. 57 (2008) 524–529
1023-3830/08/110524-6
DOI 10.1007/s00011-008-8007-8

Inflammation Research

Ivermectin inhibits LPS-induced production of inflammatory cytokines and improves LPS-induced survival in mice

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Received 13 January 2008; returned for revision 7 February 2008; received from final revision 3 April 2008; accepted by M. Parnham 4 April 2008

Published Online First 13 November 2008

Abstract. *Objective and Design*: To investigate whether ivermectin, a semi-synthetic derivative of a family of macrocyclic lactones could inhibit lipopolysaccharide (LPS)-induced inflammation in vivo and in vitro.
Materials and Methods: C57BL/6 mice were administered ivermectin (or saline) orally and challenged intraperitoneally with LPS at a lethal dose of 32 mg/kg. RAW 264.7 murine macrophages were stimulated with LPS at 1 μg/ml, with or without ivermectin for 6, 12 and 24 h. The production of tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß) and interleukin-6 (IL-6) in serum from mice and supernatants from cells were measured by ELISA. Nuclear factor-κB (NF-κB) translocation with subunit p65 was evaluated by immunocytochemical analysis.
Results: Ivermectin improved mouse survival rate induced by a lethal dose of LPS. In addition, ivermectin significantly decreased the production of TNF-α, IL-1ß and IL-6 in vivo and in vitro. Furthermore, ivermectin suppressed NF-κB translocation induced by LPS.
Conclusions: The results indicate that ivermectin may inhibit LPS-induced production of inflammatory cytokines by blocking NF-κB pathway and improve LPS-induced survival in mice. This finding might provide a new strategy for the treatment of endotoxemia and associated inflammation.

Key words: Ivermectin – Lipopolysaccharide (LPS) – Inflammatory cytokines – Nuclear factor-κB (NF-κB) – Survival

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Introduction

Macrolide antibiotics have been widely used to treat a broad array of infectious diseases. As a class of agents, they have been shown to be safe and have highly effective antibacterial activity. Furthermore, some macrolide antibiotics have been reported to modify the host immune and inflammatory responses in addition to their antibacterial activity, they are effective in the treatment and management of some inflammatory diseases [1–6]. Ivermectin is a semi-synthetic derivative of a family of macrocyclic lactones. This drug has a broad-spectrum anti-parasitic effect against various nematodes and ectoparasites [7]. It causes paralysis in many nematodes and arthropods because of its effect on ion-channels in cell membranes. Ivermectin was first used in veterinary medicine; in human studies, it has been shown to be a microfilaricid against *Onchocerca volvulus*. Most of the adverse reactions following treatment have been mild [8], earning it the title of "wonder drug" [9, 10]. The immunomodulatory effect of ivermectin in a variety of bacterial, viral, parasitic and neoplastic diseases and the influence of ivermectin on cellular and humoral immune responses have been suggested [11, 12]. Ivermectin has been shown to block or suppress LPS-induced secretion of NO and prostaglandin E2 in liver macrophages and Kupffer cells [13, 14]. These observations thus suggest that ivermectin could influence host defenses. However, the effects of this drug on RAW 264.7 murine macrophage cells and inflammatory responses in animals and on diseases associated with these responses have not been demonstrated.

Septic shock, which results from gram-negative bacterial infections, is a major cause of death among patients and animals [15]. One of the major reasons is that endotoxin or lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, induces the disturbance of immune and inflammatory responses and causes fever, hypotension and,

FOI 26-3132 - Document 5

in severe cases, resulting in tissue damage, septic shock or even death [16–19]. LPS is recognized by toll-like receptor 4 (TLR4) on cell membrane such as macrophages and triggers an acute and early release of proinflammatory mediators through TLR4-NF-κB signaling pathways that mediate host damage [20, 21]. The production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) has been shown to be important in determining the early phases of inflammation, which can progress to systemic inflammatory response syndrome [22–25].

In the present study, using RAW 264.7 murine macrophage cells and a murine endotoxic shock model, we studied first the effects of ivermectin on LPS-induced production of early inflammatory cytokines and on survival of mice induced with a lethal dose of LPS, and then the mechanism by which ivermectin exerted its effects.

Materials and methods

Reagents

Ivermectin, LPS (*Escherichia coli* 055:B5), Cy3-conjugated sheep anti-rabbit IgG, 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and other reagents for cell culture were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). Rabbit anti-NF-κB/p65 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Mice

All experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by US National Institute of Health. Female C57BL/6 mice, weighing approximately 18 to 20 g, were purchased from the Shanghai Laisite Experimental Animals Co., LTD (Certificate SCXK2003-0003). The mice were housed in microisolator cages and received food and water *ad libitum*. Laboratory temperature was 24 ± 1 °C and relative humidity was 40–80 %. Before experimentation, the mice were left to adapt to the experimental environment for 2 to 3 days.

Cell culture

RAW 264.7 murine macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). The cells were cultured in DMEM supplemented with 10 % heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate at 37 °C under a humidified atmosphere of 5 % CO_2 and 95 % air atmosphere.

Cell viability assay

Cytotoxicity studies induced by ivermectin were performed by MTT assay. RAW 264.7 cells were mechanically scraped, plated at 4×10^5 cells/ml onto 96-well plates (Costar USA) containing 100 µl of DMEM medium, and incubated overnight. Ivermectin was dissolved in DMSO; the DMSO concentrations did not exceed 0.1 % in any assay. After overnight incubation, the cells were treated with diverse concentrations of ivermectin (0–12 µg/ml) according to the experimental design. After 24 h, 50 µl of 5 mg/ml MTT in FBS-free medium was added to each well and samples were further incubated for 4 h in a humidified atmosphere at 37 °C, 5 % CO_2. MTT was removed and cells were lysed with 100 µl/well DMSO. The optical density was measured at 570 nm on a microplate reader (TECAN, Austria).

Cytokine assays in vitro

To determine the effects of ivermectin on the production of cytokines from LPS-induced cells, RAW 264.7 cells were plated onto 24-well plates (10^5 cells/well), and pretreated in the presence or absence of 0.5, 2 and 4 µg/ml of ivermectin for 1 h and then stimulated with 1 µg/ml of LPS for 6, 12, or 24 h at 37 °C, 5 % CO_2. At each period, cell-free supernatants were collected and then stored at –20 °C until assayed for cytokines. The concentrations of cytokines TNF-α, IL-1ß and IL-6 in the supernatants of RAW 264.7 cells culture were measured by sandwich enzyme-lined immunosorbent assay (ELISA) using commercially available reagents according to the manufacturer's instructions (BioLegend, Inc. Camino Santa Fe, Suite E San Diego, CA, USA).

Survival study

C57BL/6 mice were divided into five groups: the control group, administered intragastrically with normal saline; the LPS group, injected intraperitoneally with LPS at a lethal dose of 32 mg/kg; and three ivermectin + LPS groups, administered intragastrically with a single dose of ivermectin at 1, 2 and 4 mg/kg, respectively, 2 h prior to intraperitoneal injection of LPS. Mice were monitored for survival twice a day for 7 days.

Analysis of serum cytokines

Cytokines were assessed by measuring serum concentrations. Mice were randomly assigned into two groups: the LPS group, injected intraperitoneally with 32 mg/kg LPS; and the LPS + ivermectin group, administered intragastrically with a single dose of ivermectin (2 mg/kg) 2 h prior to administration of LPS. Mice in both groups were killed and their serum separated from clotted blood at 1, 3, and 6 h following administration of LPS. Serum was stored at –70 °C and concentrations of cytokine TNF-α, IL-1ß and IL-6 were measured by sandwich ELISA using commercially available reagents according to the manufacturer's instructions (BioLegend, Inc. Camino Santa Fe, Suite E San Diego, CA, USA).

Immunocytochemical analysis

The effect of ivermectin on nuclear translocation of NF-κB induced by LPS was measured by immunocytochemical analysis. RAW 264.7 cells (10^5 cells/ml) were cultured on glass cover slips, plated onto 24-well plates, and then pretreated in the presence or absence of 0.5, 2 and 4 µg/ml of ivermectin for 1 h and then stimulated with 1 µg/ml of LPS for 1 h at 37 °C, 5 % CO_2. The cells were washed with PBS and fixed in 4 % formaldehyde for 30 min, then detergent extracted with 3 % TritonX-100 for 10 min at room temperature. Cover slips were saturated with PBS containing 5 % bovine serum albumin (BSA) for 30 min at room temperature and processed for immunofluorescence with rabbit anti-NF-κB/p65 polyclonal antibody (1:50) followed by Cy3-conjugated sheep antirabbit IgG (1:100) and DAPI staining. Cover slips were mounted on slides. Fluorescence signals were analyzed by fluorescent microscopy (Olympus, Japan).

Statistical analysis

All values were expressed as mean ± SEM. Differences in survival rate of groups were assessed using the log-rank test. Differences between

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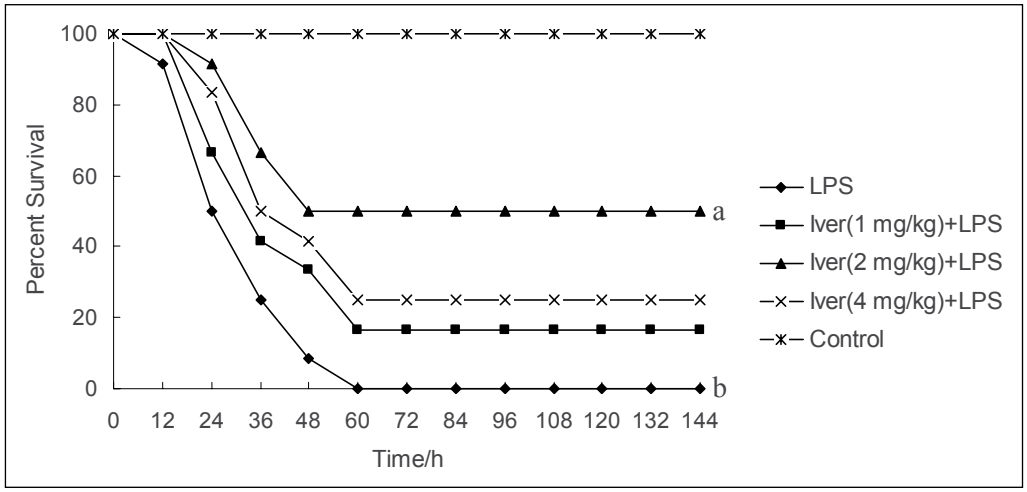


Fig. 1. Effect of ivermectin (Iver) on survival of mice induced with LPS. Mice (n = 12 for each group) were divided into control, LPS, and Iver + LPS groups. Mice in Iver + LPS groups were administered intragastrically with 1, 2 or 4 mg/kg Iver 2 h prior to intraperitoneal injection of LPS. Mice in control and LPS groups were only given normal saline or LPS. Survival was assessed every 12 h for 7 days throughout the experiment. [a]P <0.05 vs LPS group. [b]P <0.01 vs control.

mean values of normally distributed data were assessed by one-way ANOVA (Dunnett's *t*-test) and Student's *t*-test. Statistical difference was accepted at P <0.05.

Results

Ivermectin improves survival in mice challenged with lethal-dose LPS

The effect of ivermectin on LPS-induced death was assessed by measuring survival rate of mice induced with a lethal dose of LPS. The different doses of ivermectin on survival of mice induced with LPS are shown in Fig. 1. The administration of 32 mg/kg LPS resulted in 100 % mortality in mice. In contrast, when mice were given ivermectin at doses of 1, 2 and 4 mg/kg following LPS administration, survival rates were 16.7 %, 50.0 %, 25.0 %, respectively. The survival rate of mice in the ivermectin + LPS (2 mg/kg) group was significantly better than mice that only received LPS (P <0.05).

Ivermectin suppresses cytokine production in mice challenged with LPS

Early cytokine responses after LPS challenge have been well characterized and are known to occur within hours of LPS challenge. Specifically, TNF-α, IL-1ß and IL-6 have been shown to be produced early in this response and are suggested to play critical roles in driving physiological responses, however, if the cytokine production disregulated, it leads to pathological damage and septic shock. To define the effects of ivermectin on the production of cytokines in vivo that have been associated with fatal outcome, serum concentrations of these cytokines were determined at different time points following LPS administration. As shown in Fig. 2, mean concentrations of cytokines in the LPS + ivermectin (2 mg/kg) group were significantly different from that of the LPS group at 1 h for TNF-α (P <0.05) (Fig. 2A), at 3 h and 6 h for IL-1ß (P <0.01) (Fig. 2B) and at 3 h and 6 h for IL-6 (P <0.05 or <0.01) (Fig. 2C). Ivermectin significantly

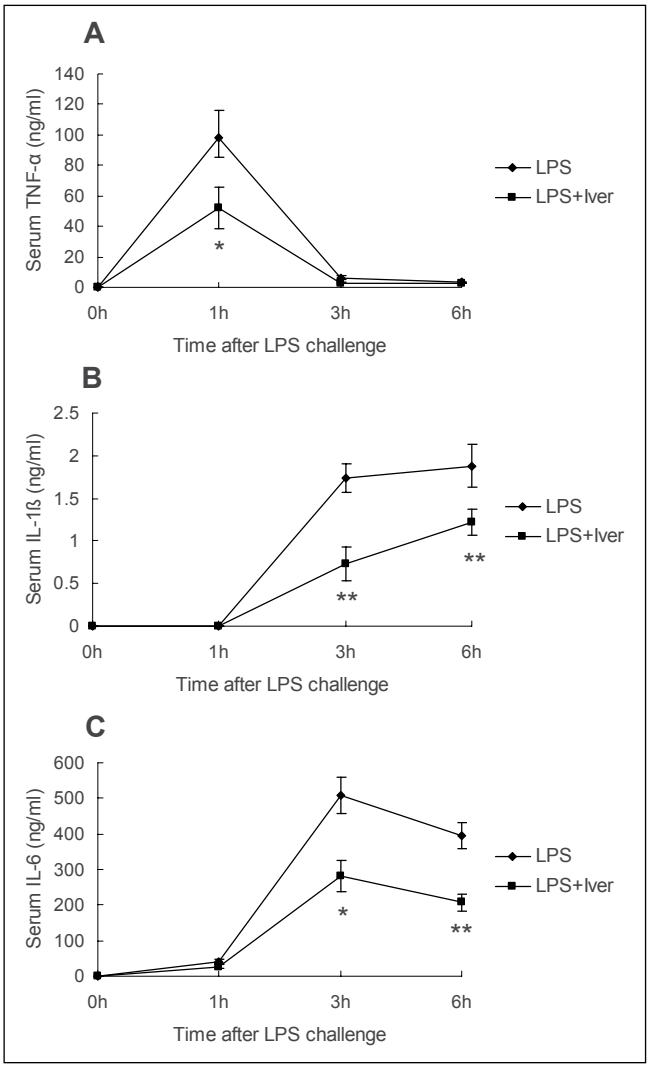


Fig. 2. Effect of ivermectin (Iver) on serum TNF-α, IL-1β and IL-6 induced by LPS. Mice were given Iver (2 mg/kg) or normal saline 2 h prior to intraperitoneal injection of LPS. Serum concentrations of TNF-α, IL-1β and IL-6 in LPS and LPS + Iver groups were measured at 1, 3 and 6 h following LPS challenge (n = 16 for each group). Mean ± SEM. *P <0.05, **P <0.01 vs LPS.

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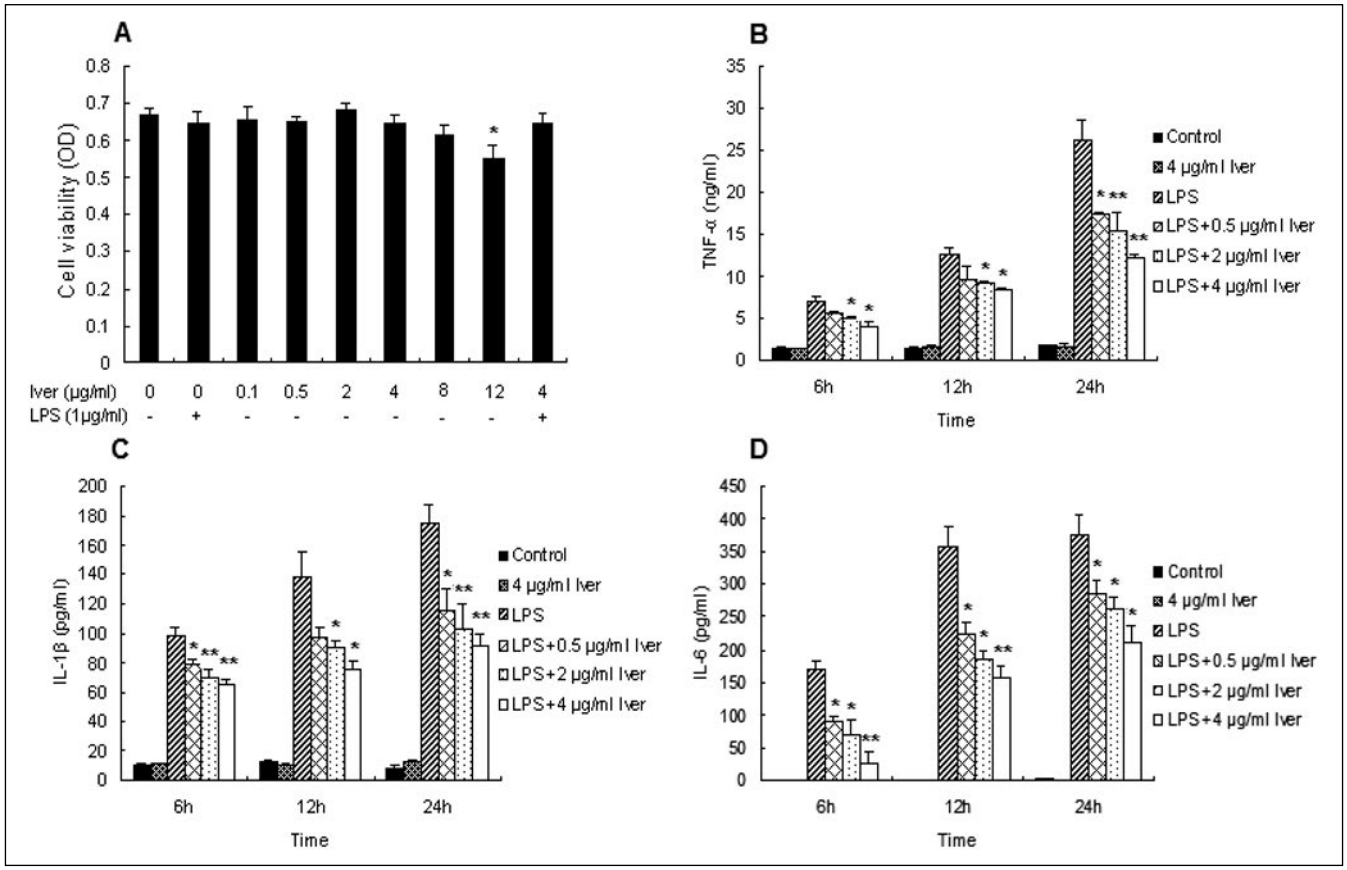


Fig. 3. Effects of Ivermectin on the viability of RAW 264.7 cells and production of TNF-α, IL-1ß and IL-6 in LPS-induced RAW 264.7 cells. (A) Effect of Ivermectin (Iver) on the viability of RAW 264.7 cells. RAW 264.7 cells were incubated in the presence or absence of 0.1-12 μg/ml Iver for 24 h. Cell viabilities were determined by MTT assay. Data are presented as the means ± SEM of three independent experiments. *P <0.05 vs control. (B, C, D) Effect of different concentrations of Ivermectin (Iver) on production of TNF-α, IL-1ß and IL-6 in LPS-induced RAW 264.7 cells. The cells were treated with 1 μg/ml LPS alone or LPS plus different concentrations (0.5, 2, 4 μg/ml) of Iver for 6, 12 and 24 h. Control values were obtained in the absence of LPS or Iver. The values are the means ± SEM of three independent experiments. *P <0.05, **P <0.01 vs LPS group.

reduced TNF-α response at 1 h, IL-1ß and IL-6 responses at 3 h and 6 h. In addition to the concentrations in serum, TNF-α, IL-1ß and IL-6 were nearly undetectable in the control group and ivermectin-only group (data not shown).

Effects of ivermectin on cell viability

The cytotoxic effect of ivermectin on RAW 264.7 cells was initially evaluated. The cells were treated with various concentrations of ivermectin ranging from 0.1 to 12 μg/ml for 24 h. Ivermectin at concentrations from 0.1 to 8 μg/ml had no effect on cell survival. However, the viability of the cells was reduced when 12 μg/ml of ivermectin was used (Fig. 3A). In all other cell experiments, the concentrations of ivermectin applied never exceeded 8 μg/ml. Ivermectin (4 μg/ml) alone and in the presence of LPS (1 μg/ml) did not further affect the viability of RAW 264.7 cells (Fig. 3A). These results confirmed that the effects of ivermectin on RAW 264.7 cells were not due to a reduction in cell viability in each condition.

Ivermectin suppresses LPS-induced cytokine production in RAW 264.7 cells

TNF-α, IL-1ß and IL-6 concentrations in the culture supernatant of RAW 264.7 cells were measured by sandwich ELISA (Figs. 3B, C and D). Treatment of RAW 264.7 cells with LPS alone resulted in a significant increase in accumulation of each of the cytokines as compared to that in controls (P <0.01) after incubation for 6 h, 12 h and 24 h. However, in a dose-dependent manner at each time period, TNF-α, IL-1ß and IL-6 levels in the supernatants of cells treated with LPS plus 0.5, 2 and 4 μg/ml of ivermectin significantly decreased compared to the LPS group (P <0.05 or 0.01).

Ivermectin blocks LPS-induced NF-κB translocation in RAW 264.7 cells

We next evaluated the effect of ivermectin on LPS induction of the NF-κB pathway. We first analyzed the subcellular distribution of p65 by immunofluorescence and found that p65 was distributed in the cytoplasmic compartment in

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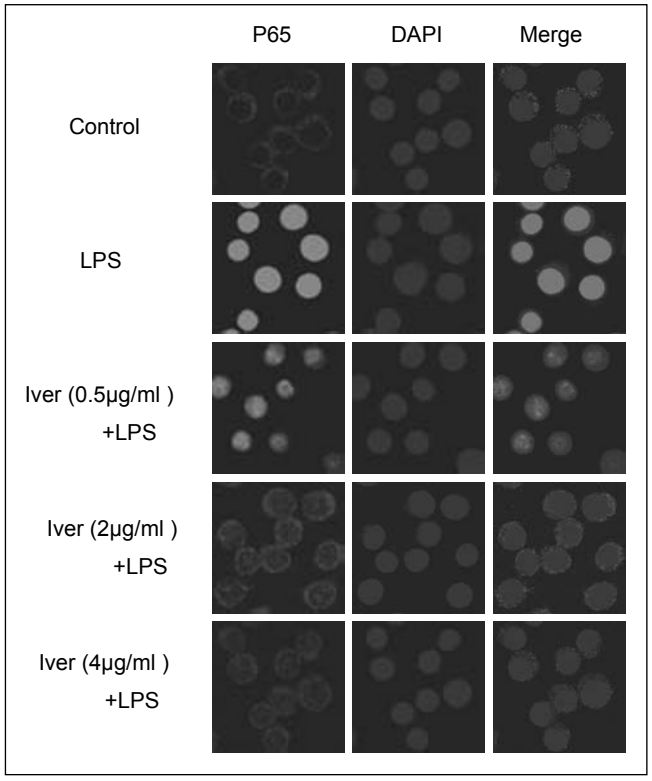


Fig. 4. Effect of ivermectin (Iver) on LPS-induced NF-κB translocation by immunocytochemistry. The cells were pretreated with Iver (0.5, 2, 4 μg/ml) for 1 h and stimulated for 1 h with LPS, then were fixed, permeabilized, and incubated with rabbit anti-NF-κB/p65 polyclonal antibody followed by Cy3-conjugated anti-rabbit IgG (red). The nuclei of the corresponding cells were demonstrated by DAPI staining (blue). Magnification for images was 600×.

all cells before LPS stimulation. One hour after stimulation with LPS (1 μg/ml), most intracellular p65 was translocated from the cytoplasm to the nucleus, demonstrated with strong p65 staining in the nucleus. However, in a dose-dependent manner, nuclear translocation of p65 induced by LPS was dramatically inhibited in cells cultured with ivermectin (Fig. 4).

Discussion

Macrophages are one of the primary innate immune cell types that recognize pathogen-associated molecular patterns such as LPS in the case of gram-negative bacterial infections and trigger innate immune response through TLR signaling. Binding of LPS to TRL4 receptor activates an intracellular tyrosine kinase system that eventually leads to activation of a redox-sensitive transcription factor NF-κB, which in turn induces expression of a number of inflammatory mediators including TNF-α, IL-1β and IL-6 [20]. Proinflammatory cytokines (TNF-α, IL-1β and IL-6) have been suggested to induce tissue damage and multiple organ failure, are considered to be an important initiator of the inflammatory response and to mediate the development of various inflammatory diseases [26–29]. In the present study, we demonstrate for the first time that ivermectin inhibits TNF-α, IL-1β and IL-6 production in a dose-dependent manner in LPS-induced RAW 264.7 macrophage cells (Figs. 3B, C and D).

LPS-induced murine septic shock model is widely used in the search for drugs and treatment tools for sepsis caused by gram-negative bacterial infections. We used a murine endotoxic shock model and studied the effect of ivermectin on survival of mice induced with a lethal dose of LPS. Ivermectin improved the survival rate of mice (Fig. 1), and the results indicate that ivermectin can consistently prevent LPS-induced death in mice. However, at a dose of 4 mg/ml, the survival rate tended to be lower, suggesting that ivermectin may be toxic at higher concentrations according to the toxic dose (LD_{50}) of 50–60 mg/kg for p.o. ivermectin in normal mice [30].

In the survival study, we demonstrated that treatment with ivermectin at a dose of 2 mg/kg produced the best inhibitory effects on the mortality rate of mice induced with LPS. Thus, we observed the effects of ivermectin at this dosage on serum TNF-α, IL-1β and IL-6 production in a LPS-induced mouse model. As we expected, ivermectin inhibited serum TNF-α, IL-1β and IL-6 production, and was consistent with our results in vitro (Fig. 2). Ivermectin-mediated reductions in serum TNF-α, IL-1β and IL-6 production were considered to contribute to improved mouse survival.

In order to further explore the mechanisms by which ivermectin exerted its effects, we investigated the effect of ivermectin on LPS-induced NF-κB translocation. NF-κB has been evidenced to play a major role in LPS-induced expression of inflammatory cytokines including TNF-α, IL-1β and IL-6 in macrophages [31, 32]. NF-κB participates in regulating the expression of cytokines and other mediators that are involved in the inflammatory response [33]. In the present study, we clearly found that the NF-κB factor p65, which was translocated from the cytoplasm to the nucleus by LPS exposure, was strongly inhibited by ivermectin in a dose-dependent manner (Fig. 4). Accordingly, we conclude that the inhibition of ivermectin on LPS-induced inflammatory cytokine production and the improvement of survival in mice with endotoxic shock are at least partially mediated by the suppression of the NF-κB pathway.

In summary, our study demonstrates that ivermectin markedly suppresses cytokine production in vitro and in vivo, may be mediated by suppressing NF-κB pathway, and consequently improves survival in endotoxic shock. Thus, ivermectin may prove to be a valuable protective agent against septic shock.

Acknowledgments. This work was supported by the National Science Foundation of China (No. 30671586).

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From:	s22
To:	s22 ; Streamlined Submission; s22
Cc:	Commercial Eyes Regulatory
Subject:	RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input [SEC=OFFICIAL]
Date:	Tuesday, 18 July 2023 3:00:00 PM
Attachments:	image006.jpg image007.png image008.jpg image002.png

Hi s22

With regard to the queries on Ziverdox, in your emails dated 30 June 2023 and 10 July 2023, please see the following comments from the TGA.

Nonclinical

In vivo PK data is required to determine whether the pharmacokinetic profiles in the laboratory animal species (particularly those used in the pivotal repeat-dose toxicity studies) were sufficiently similar to allow them to serve as appropriate models for the assessment of drug toxicity in humans.

Drug interactions between the 3 ingredients and between the drug combination and other drugs, which may be administered with Ziverdox will assist in determining potential interactions in humans as per CPMP/EWP/560/95/Rev. 1 Corr. 2 (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf).

Clinical

(queries in email of 30 Jun)

Suggest there needs to be tightly defined inclusion criteria – eg mild to moderately severe COVID-19 (how exactly will this be defined) at risk of progression. There should also be detailed knowledge of why there is an increased risk of progression to severe disease. Suggest that preferential inclusion of older patients makes the most sense – we know that most younger people do not progress to serious disease. There needs to be record of vaccination status, and previous COVID-19 illness. It is also not clear as to how the length of follow up is determined, and how long before the start of medication the person can have had symptoms etc. A double blind RCT is an appropriate design – but there needs to be careful attention to ensure blinding is maintained. It is noted there is no discussion regarding the current antivirals (eg Paxlovid/ molnupiravir) and whether these are to be an exclusion criteria.

There needs to be careful consideration as to avoiding any perceived or real conflicts of interest. It is unclear who the approving ethics committee will be.

It remains unclear at this time as to the FDC justification as per the advice provided by TGA on 31 Oct 2022. s47

(queries in email of 10 Jul)

For Q.1 - s47 would likely be appropriate.

For Q.2 - As previously advised by the TGA (e.g. email dated 6 Apr 2023), the TGA does not typically provide advice on the clinical study design.

Regards,
s22

s22
Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
s22
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Monday, 10 July 2023 11:52 AM
To: s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input

Hi s22

Thank you again for considering our client's request for any feedback regarding the clinical and nonclinical development of the triple therapy Ziverdox, for the treatment of COVID-19.

s47

Could I please ask TGA to also comment on:

s47

Thank you very much in advance. Our client is appreciative of any advice TGA are able to offer and welcome the opportunity to discuss at any time that is suitable.

Regards,

s22
Innovative Therapeutics Manager, Regulatory, Quality & Compliance

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From: s22 @health.gov.au>
Sent: Tuesday, July 4, 2023 9:54 AM
To: s22 @commercialeyes.com.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
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Hi s22

TGA is aiming to provide a response to the queries by mid-July.

Regards,
s22

Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA

Australian Government, Department of Health and Aged Care
s22
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
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From: s22 @commercialeyes.com.au>
Sent: Friday, 30 June 2023 11:32 AM
To: Streamlined Submission <streamlinedsubmission@health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: Topelia - Ziverdox triple therapy for COVID-19 - request for input

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Dear Madam/Sir,

On behalf of our client Topelia Pty Ltd, Commercial Eyes Pty Ltd (CEPL) seek TGA advice on the nonclinical and clinical development program for "ZIVERDOX" s47 containing the active ingredients ivermectin, doxycycline hyclate (hydrochloride) and zinc citrate dihydrate.

The proposed indication for ZIVERDOX is:

- *The treatment of mild to moderate symptomatic adult patients diagnosed with COVID-19 caused by SARS-CoV-2 virus.*

Background

Topelia Pty Ltd initially proposed to support the registration of ZIVERDOX via a literature-based submission (LBS). Regulatory activities during the preparation of the LBS included request for a pre-submission meeting with the TGA (written advice received 7 April 2022), seeking TGA approval on the proposed literature search strategy (TGA advice received 25 August 2022) and submission of a justification for the s47 (TGA feedback received 6 April 2023).

Taking into consideration the feedback from the TGA, Topelia Pty Ltd now propose to support the registration of ZIVERDOX with nonclinical and clinical studies as applicable.

The proposed nonclinical and clinical program includes the following studies:

Nonclinical studies

Pharmacokinetics

s47 [redacted]

Toxicology:

s47 [redacted]

Clinical studies

s47 [redacted]

Specific questions

s47 [redacted]

Our client would appreciate any feedback TGA can offer on the above.
Thank you in advance.

Regards,

s22 [redacted]
Innovative Therapeutics Manager, Regulatory, Quality & Compliance

Level 19, 15 William Street, Melbourne VIC 3000
Mailing Address: PO Box 620 Collins St. West, VIC 8007

s22

www.commercialeyes.com.au

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Pages 7-63 have been removed as they are exempt from release according to section 47 of the FOI Act.

From: s22 on behalf of Streamlined Submission
To: "Streamlined Submission"; s22 "
Cc: "Commercial Eyes Regulatory"
Subject: RE: Stream 2 COVID E22-551893 RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input [SEC=OFFICIAL]
Date: Tuesday, 28 November 2023 5:21:09 PM
Attachments: image001.png
image003.png

Hi s22 ,

Thanks again for your enquiry,
To assist the Clinical team with providing accurate advice, could the sponsor please confirm the following:

Can the Sponsor please define which population they intend to treat:

- *To treat mid-moderate covid in generally healthy population who are at lower risk for disease progression?*
- *To treat mild moderate covid in the population who are at high risk for disease progression from mild moderate covid to severe covid?*

What is their proposed indication and what is the efficacy endpoint (outcome measure) to be studied

- *to reduce the duration of covid symptom*
- *to reduce the rate of hospitalisation and death (in high risk population)?*

Kind regards
s22

Case Manager - Application and Advisory Management
Prescription Medicines Authorisation Branch

Medicines Regulation Division | Health Products Regulation Group
Australian Government Department of Health
s22 E: streamlinedsubmission@health.gov.au
Location: Symonston ACT
PO Box 100, Woden ACT 2606, Australia

The Department of Health acknowledges the Traditional Custodians of Australia and their continued connection to land, sea and community. We pay our respects to all Elders past and present.
This response is general information given to you without prejudice; it is not binding on the TGA and you should get your own independent legal advice to ensure that all of the legislative requirements are met

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From: s22 @health.gov.au> **On Behalf Of** Streamlined Submission
Sent: Friday, 24 November 2023 1:47 PM
To: s22 @commercialeyes.com.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: Stream 2 COVID E22-551893 RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input [SEC=OFFICIAL]

Hi s22 ,

Thanks very much for your email. I have forwarded your follow up questions onto the relevant teams for consideration and will revert back once a consolidated response is available,

Have a great weekend,
Kind regards
s22

Case Manager - Application and Advisory Management
Prescription Medicines Authorisation Branch

Medicines Regulation Division | Health Products Regulation Group
Australian Government Department of Health
s22 | E: streamlinedsubmission@health.gov.au
Location: Symonston ACT
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From: s22 @commercialeyes.com.au>
Sent: Thursday, 23 November 2023 1:49 PM
To: s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input

Hi s22

Thank you again for all the assistance TGA has provided to date on the queries relating to the development of our client's medicine, Ziverdox.

s47

s47

Any assistance TGA could offer would be greatly appreciated. Thank you.

Regards,

s22
Innovative Therapeutics Manager, Regulatory, Quality & Compliance

CommercialEyes

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From: s22 @health.gov.au>
Sent: Tuesday, July 18, 2023 3:01 PM
To: s22 @commercialeyes.com.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input [SEC=OFFICIAL]

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Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
s22
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

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From: s22 @commercialeyes.com.au>
Sent: Monday, 10 July 2023 11:52 AM
To: s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input

Hi s22 ,

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Could I please ask TGA to also comment on:

s47

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Innovative Therapeutics Manager, Regulatory, Quality & Compliance

CommercialEyes

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From: s22 @health.gov.au>
Sent: Tuesday, July 4, 2023 9:54 AM
To: s22 @commercialeyes.com.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input [SEC=OFFICIAL]

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Regards,
s22

Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
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Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
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From: s22 @commercialeyes.com.au>
Sent: Friday, 30 June 2023 11:32 AM
To: Streamlined Submission <streamlinedsubmission@health.gov.au>; Streamlined Submission

<streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: Topelia - Ziverdox triple therapy for COVID-19 - request for input

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Dear Madam/Sir,

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Nonclinical studies

Pharmacokinetics

s47

Toxicology:

s47

Clinical studies

s47

s47 [redacted]

Specific questions

s47 [redacted]

Our client would appreciate any feedback TGA can offer on the above.
Thank you in advance.

Regards,

s22 [redacted]
Innovative Therapeutics Manager, Regulatory, Quality & Compliance

CommercialEyes

Level 19, 15 William Street, Melbourne VIC 3000
Mailing Address: PO Box 620 Collins St. West, VIC 8007
s22 [redacted]
www.commercialeyes.com.au

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From: s22
To: s22 ; Streamlined Submission; s22
Cc: Commercial Eyes Regulatory
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input
Date: Thursday, 29 February 2024 3:14:33 PM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.jpg
image006.jpg
Nonclinical development studies Ziverdox.pdf

Hi s22 ,

On behalf of our client, Topelia Pty Ltd, thank you again for all the advice provided on the development of the triple therapy Ziverdox, for use in COVID patients.

In response to the valuable feedback from TGA regarding the nonclinical data requirements, our client has prepared a study protocol to generate data to satisfy the requirements.

We respectfully seek TGA's review and any feedback on the attached proposed studies that have been designed to align with TGA's feedback on the nonclinical data requirements for Ziverdox.

Please do not hesitate to advise if anything requires further clarification or if a phone call would assist.

Thank you.

Regards,

s22
Innovative Therapeutics Manager, Regulatory, Quality & Compliance

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From: s22 @health.gov.au>
Sent: Tuesday, July 18, 2023 3:01 PM

To: s22 @commercialeyes.com.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input [SEC=OFFICIAL]

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s22

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Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA
Australian Government, Department of Health and Aged Care
s22
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
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Sent: Monday, 10 July 2023 11:52 AM
To: s22 @health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input

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Could I please ask TGA to also comment on:

s47

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Sent: Tuesday, July 4, 2023 9:54 AM
To: s22 @commercialeyes.com.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: RE: Topelia - Ziverdox triple therapy for COVID-19 - request for input [SEC=OFFICIAL]

CAUTION: This email is external and originated from outside Commercial Eyes. Do not click on links or open attachments unless you recognise the sender and sender's email address and know the content is safe.

Hi s22

TGA is aiming to provide a response to the queries by mid-July.

Regards,
Nicolle

s22
Assistant Director - Application and Advisory Management
Prescription Medicines Authorisation Branch
Medicines Regulation Division | TGA

Australian Government, Department of Health and Aged Care
s22
Location: TGA, 27 Scherger Drive, Fairbairn ACT 2609
PO Box 100, Woden ACT 2606, Australia

The Department of Health and Aged Care acknowledges the traditional owners of country throughout Australia, and their continuing connection to land, sea and community. We pay our respects to them and their cultures, and to elders both past and present.

This response is general information given to you without prejudice; it is not binding on the TGA and you should get your own independent legal advice to ensure that all of the legislative requirements are met.

Please note that this email is intended only for the use of the addressee and may contain confidential or legally privileged information. If you receive this email in error, please notify the sender immediately and delete all copies of this email.

From: s22 @commercialeyes.com.au>
Sent: Friday, 30 June 2023 11:32 AM
To: Streamlined Submission <streamlinedsubmission@health.gov.au>; Streamlined Submission <streamlinedsubmission@health.gov.au>; s22 @health.gov.au>
Cc: Commercial Eyes Regulatory <regulatory@commercialeyes.com.au>
Subject: Topelia - Ziverdox triple therapy for COVID-19 - request for input

REMINDER: Think before you click! This email originated from outside our organisation. Only click links or open attachments if you recognise the sender and know the content is safe.

Dear Madam/Sir,

On behalf of our client Topelia Pty Ltd, Commercial Eyes Pty Ltd (CEPL) seek TGA advice on the nonclinical and clinical development program for "ZIVERDOX" s47 containing the active ingredients ivermectin, doxycycline hyclate (hydrochloride) and zinc citrate dihydrate.

The proposed indication for ZIVERDOX is:

- *The treatment of mild to moderate symptomatic adult patients diagnosed with COVID-19 caused by SARS-CoV-2 virus.*

Background

Topelia Pty Ltd initially proposed to support the registration of ZIVERDOX via a literature-based submission (LBS). Regulatory activities during the preparation of the LBS included request for a pre-submission meeting with the TGA (written advice received 7 April 2022), seeking TGA approval on the proposed literature search strategy (TGA advice received 25 August 2022) and submission of a justification for the s47 (TGA feedback received 6 April 2023).

Taking into consideration the feedback from the TGA, Topelia Pty Ltd now propose to support the registration of ZIVERDOX with nonclinical and clinical studies as applicable.

The proposed nonclinical and clinical program includes the following studies:

Nonclinical studies

Pharmacokinetics

s47

Toxicology:

s47

Clinical studies

s47

Specific questions

s47

Our client would appreciate any feedback TGA can offer on the above.
Thank you in advance.

Regards,

s22

Innovative Therapeutics Manager, Regulatory, Quality & Compliance

Level 19, 15 William Street, Melbourne VIC 3000
Mailing Address: PO Box 620 Collins St. West, VIC 8007

s22

www.commercialeyes.com.au

Disclaimer:

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Pages 9-16 have been removed as they are exempt from release according to section 47 of the FOI Act.

From: TGA Exports
To: s22 @topeliaaustralia.com
Cc: s22
Subject: Ivermectin/Doxycycline/Zinc Chelate - Ziverdox- Topelia Australia Limited- s47 - EXP22/6051819- 3.10.22 - Approval [SEC=OFFICIAL]
Date: Wednesday, 16 November 2022 10:18:12 AM
Attachments: Ivermectin Doxycycline Zinc Chelate - Ziverdox- Topelia Australia Limited- s47 ~ Approval.pdf
image001.png
image002.gif

Good Morning,
Please see approval.
Kind Regards,

Application Entry, Support and Export Section

Prescription Medicines Authorisation Branch | Medicines Regulation Division
Health Products Regulation Group (Therapeutic Goods Administration)
Australian Government Department of Health
E: **tga.exports@health.gov.au**
PO Box 100, WODEN ACT 2606, Australia

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Australian Government

Department of Health and Aged Care

Therapeutic Goods Administration

s22
Director
Topelia Australia Limited
Level 1 229 Great Northern Road
Five Dock NSW 2046
Email: s22 @topeliaaustralia.com

E22-67322

Dear s22 ,

APPROVAL REF NO: EXP22/6051819
RE: REQUEST FOR APPROVAL TO EXPORT THERAPEUTIC GOODS
TO s47 FOR
CLINICAL TRIAL USE

I refer to your correspondence dated **03 November 2022** requesting approval to export clinical trial good(s) to a distribution centre in the following country(ies) for use in an ICH Good Clinical Practice (GCP) compliant study, **Protocol No:** s47 .

Country(ies)
s47

The good(s) to be exported:

- **Ivermectin/Doxycycline/Zinc Chelate, Ziverdox,** s47

I note you have provided me with written assurances from **Dr David Fox Chief Executive Officer** on behalf of Topelia Australia Limited, the sponsor of this study. These written assurances state, amongst other things, that the trial will be conducted in accordance with the ICH GCP and that the trial sponsor will comply with any requests from the TGA for information about the trial. As exporter you have also provided assurances that the goods have been manufactured under standards that comply with Good Manufacturing Practice.

Pursuant to Section 19(1) of the *Therapeutic Goods Act 1989*, approval is granted for the export from Australia of the above mentioned therapeutic good(s) for use in clinical trials in humans. This approval is subject to the following conditions:

1. This approval is for the export of the good(s), as detailed above, from **Australia** to s47 .

2. These goods are solely for experimental purposes in humans as per the Clinical Trial Protocol No: s47 .

3. This approval is valid for the export of multiple consignments of the good(s) to the abovementioned site(s), **within 12 (twelve) months from the date of this letter**.

4. If any manufacturing steps occur at an Australian manufacturing site, the site must hold acceptable evidence of a Licence to Manufacture Therapeutic Goods issued by the TGA.

5. The Exporter will comply with all relevant laws of any other state or national government and any relevant international requirements pertaining to the exportation, importation supply and use of this product.

6. A record relating to the source of the supply, distribution and disposal of this unregistered product is kept and if requested by the Secretary of this Department, these records must be supplied.

7. The Therapeutic Goods Administration can provide no assurance as to the quality, safety and efficacy in the proposed usage. It should be noted that the proposed clinical use of this product must be regarded both medico-legally and ethically as experimental.

8. The Australian Government accepts no responsibility for any defects in these goods related to manufacture, distribution or directions for use, including dosage or any other matter related to their use.

9. A report detailing the quantity of goods that have been exported to the distribution centre must be submitted to the TGA every six months. Please note that the first report required will be for the period ending **30 Demeber 2022.** The report should be submitted in the format set out in Attachment 1.

Request for reconsideration of an initial decision
This decision is a reviewable initial decision under section 60 of the Act. Under section 60, a person whose interests are affected by a 'reviewable' initial decision, can seek reconsideration of the initial decision.

As this document constitutes written notice of the making of an initial decision being given by the Secretary, a request for reconsideration of this initial decision must be given to the Minister in writing within 90 (calendar) days after the initial decision notice is given and be accompanied by any information that you wish to have considered by the Minister. A request for reconsideration given to the Minister outside the statutory 90 day reconsideration period cannot be accepted.

The Minister may either personally undertake a request for reconsideration of an initial decision or delegate this function to an officer of the Department with the appropriate delegation.
Under section 60(3A) of the Act, the Minister (or the Minister's delegate) is not able to consider any information provided after the making of a request for reconsideration of an initial decision unless the information is provided in response to a request from the Minister (or the Minister's delegate), or it is information that indicates that the quality, safety or efficacy of the relevant therapeutic goods is unacceptable.

Guidelines for requesting reconsideration of an initial decision
Prior to requesting reconsideration of an initial decision, persons affected by an initial decision are advised to refer to the TGA website <https://www.tga.gov.au/reconsideration-reviewable-initial-decisions> for specific information and detailed guidance for making a request for reconsideration. A request for reconsideration should then be made in writing, signed and dated by the person requesting reconsideration and should include the following:

- a copy of the initial decision notification letter, i.e. this letter (or other evidence of notification);
- identify, and describe with as much specificity as possible, which component(s) of the initial decision should be reconsidered and set out the reasons why reconsideration is requested;
- any information/documentation in support of the request, clearly labelled to correspond with (any or each of) the reasons why reconsideration is requested; and
- an email address nominated for the purposes of receiving correspondence in relation to the request for reconsideration.

All requests for reconsideration should be given to the Minister by email:

Email: '**decision.review@health.gov.au**'

Subject: "**<insert name of person/company making request>** - Request for Reconsideration Under Section 60 of the *Therapeutic Goods Act 1989*"

Requests for reconsideration that include material which cannot be attached to a single email, may be submitted under multiple, sequentially numbered emails (e.g. "… - Email 1 of 3", "… - Email 2 of 3" etc). All sequentially numbered emails must be given to the Minister on the same date.

Under section 60 of the Act, the decision upon reconsideration by the Minister (or the Minister's delegate) must be to either 'confirm', 'revoke' or 'revoke and substitute' the initial decision. The Minister (or the Minister's delegate) must give notice in writing of the outcome of the decision upon reconsideration to the person whose interests are affected, within 60 (calendar) days after making a request for reconsideration. If the Minister (or the Minister's delegate) fails to give such notice within 60 days, the Minister (or the Minister's delegate) is deemed to have confirmed the initial decision. Subject to the *Administrative Appeals Tribunal Act 1975* (AAT Act), if you are dissatisfied with the decision upon reconsideration by the Minister (or the Minister's delegate), you can apply to the Administrative Appeals Tribunal (AAT) for a review of that decision upon reconsideration.

NOTE: This initial decision remains in effect unless and until it is revoked or revoked and substituted by the Minister (or the Minister's delegate) as a result of a request for reconsideration under section 60 of the Act OR is set aside, varied or remitted by the AAT or is otherwise overturned or stayed.

If you require any further assistance concerning this matter please contact me by telephone on s22

Yours sincerely,

Signed and authorised by
s22
Delegate of the Secretary

16 November 2022

Attachment 1

Australian Government
Department of Health
Therapeutic Goods Administration

TGA use only

This form, when completed, will be classified as '**For official use only**'.
For guidance on how your information will be treated by the TGA see: Treatment of information provided to the TGA at <https://www.tga.gov.au/treatment-information-provided-tga>.

Six monthly report

Export of unregistered therapeutic good(s)

Six monthly report by the applicant, approved under Subsection 19(1)(b) of the *Therapeutic Goods Act 1989*.

Protocol No.:	s47
Company name:	**Topelia Australia Limited**
Original approval letter Ref. No.:	**EXP22/6051819**

Report for the six month period ending:

☐ **30 June** *(year: yyyy)* ☐ **31 December** *(year: yyyy)*

Details of specified goods exported

Clinical trial good(s) and presentation (e.g. kit)	Recipients name (e.g. PI)	Site name/ Distribution centre	Address (including country)	Total No. of units exported

Signed on behalf of exporter: | Date:

*** NB: Please email the completed six monthly report forms to: eps@tga.gov.au***