BRIEF REPORT

The IMPDH inhibitor merimepodib provided in combination with the adenosine analogue remdesivir reduces SARS-CoV-2 replication to undetectable levels *in vitro* [version 1; peer review: 2 approved with reservations]

Natalya Bukreyeva¹, Rachel A. Sattler¹, Emily K. Mantlo¹, Timothy Wanninger¹, John T. Manning ¹, Cheng Huang¹, Slobodan Paessler¹, Jerome B. Zeldis²

¹Department of Pathology, University of Texas Medical Branch at Galveston, Galveston, Texas, 77555, USA

²ViralClear, subsidiary of BioSig, Inc., Westport, Connecticut, 06880, USA

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the novel coronavirus responsible for the ongoing COVID-19 pandemic, which has resulted in over 2.5 million confirmed cases and 170,000 deaths worldwide as of late April 2020. The pandemic currently presents major public health and economic burdens worldwide. No vaccines or therapeutics have been approved for use to treat COVID-19 cases in the United States despite the growing disease burden, thus creating an urgent need for effective treatments. The adenosine analogue remdesivir (REM) has recently been investigated as a potential treatment option, and has shown some activity in limiting SARS-CoV-2 replication. We previously reported that the IMPDH inhibitor merimepodib (MMPD) provides a dose-dependent suppression of SARS-CoV-2 replication *in vitro*. Here, we report that a 4-hour pre-treatment of Vero cells with 2.5 μM MMPD reduces the infectious titer of SARS-CoV-2 more effectively than REM at the same concentration. Additionally, pre-treatment of Vero cells with both REM and MMPD in combination reduces the infectious titer of SARS-CoV-2 to values below the detectable limit of our TCID₅₀ assay. This result was achieved with concentrations as small as 1.25 μM MMPD and 2.5 μM REM. At concentrations of each agent as low as 0.31 μM, significant reduction of viral production occurred. This study provides evidence that REM and MMPD administered in combination might be an effective treatment for COVID-19 cases.

Keywords

merimepodib, remdesivir, SARS-CoV-2, COVID-19

Open Peer Review

Approval Status ? ?

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1. Luka Cicin-Sain , Helmholtz Centre for Infection Research, Braunschweig, Germany		
2. Mahmoud Kandeel , King Faisal University, Al-Ahsa, Saudi Arabia Kafrelsheikh University, Kafrelsheikh, Egypt		
Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the **Emerging Diseases** and **Outbreaks** gateway.

This article is included in the **Coronavirus** collection.

Corresponding author: Jerome B. Zeldis (jbzeldis1950@gmail.com)

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Competing interests: Jerome B. Zeldis is the executive chair of ViralClear Pharmaceuticals, and developed merimepodib. Slobodan Paessler serves as a consultant to ViralClear.

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Introduction

The novel coronavirus SARS-CoV-2 is responsible for the current coronavirus disease 2019 (COVID-19) pandemic that originated in Wuhan, China in December of 2019. Since its emergence, the virus has infected 2.5 million people and is responsible for 170,000 deaths at the time of writing. In addition to the significant health burden, the pandemic is causing a worldwide economic crisis as countries attempt to limit the spread of SARS-CoV-2. There is a dire need for efficacious treatments, as the United States has yet to approve a vaccine or therapeutic to combat COVID-19. The most efficient means of approval involves drugs with a history of clinical testing. Two of these drugs, remdesivir (REM) and merimepodib (MMPD), have already been tested in patients. REM is currently in clinical trials as a COVID-19 therapeutic, while MMPD has previously been tested against chronic hepatitis C and psoriasis with an excellent safety profile^{1,2}.

REM is an adenosine analogue that displays broad-spectrum antiviral activity against RNA viruses, and has been developed for the treatment of Ebola virus disease³. The drug causes a delayed chain termination of RNA synthesis roughly five nucleotides downstream of the incorporated nucleotide analogue⁴. REM has demonstrated antiviral effects against both Ebola and Nipah viruses in nonhuman primates (NHPs)^{5,6}, and has recently shown efficacy as a COVID-19 therapeutic in NHPs⁷. However, the drug must be administered intravenously limiting the ability for use outside of a hospital setting.

MMPD is a selective and potent inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH), an enzyme involved in de novo guanine nucleotide synthesis⁸, which manipulates cellular nucleoside triphosphate levels to control the rapid replication of viruses. The drug has been tested for *in vitro* inhibition of many RNA viruses including Ebola, Junin, Lassa, Zika, and Chikungunya viruses⁹. We recently determined that MMPD suppresses the replication of SARS-CoV-2 by 3-log when Vero cells were pre-treated 4 hours prior to infection¹⁰.

In this study, we aimed to compare the antiviral effect of REM to MMPD in a tissue culture model in which cells were pre-treated with either agent prior to SARS-CoV-2 infection, and to determine whether pre-treatment with both REM and MMPD could further reduce the replication of SARS-CoV-2 *in vitro*.

Methods

Cells and viruses

Vero cells (ATCC Cat# CCL-81, RRID:CVCL_0059) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (HyClone). SARS-CoV-2 USA-WA1/2020 was obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), which obtained the original isolate from the Centers for Disease Control and Prevention (CDC). Upon obtaining the isolate, virus titration was performed. A subsequent low multiplicity of infection (MOI) passage was performed in Vero cells (DMEM supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin), and the resulting stock was titrated

prior to use. All experiments were conducted within approved biosafety level three (BSL-3) laboratories at the University of Texas Medical Branch (UTMB). All personnel undergo routine medical surveillance.

Compound treatment and infection of Vero cells

MMPD clinical lot #2 (VX-497 Bulk Drug Substance # 300445), which is supplied as a white solid, was obtained from the PCI (Newburyport, MA). 10 mM stock of MMPD was prepared in DMSO and stored at -80°C. REM (Synonym GS-5734), Cat. # HY-104077, which is supplied as 10 mM solution in DMSO, was obtained from MedChemExpress (Monmouth Junction, NJ) and stored at -80°C. Both MMPD and REM then were diluted in compound dilution media to reach the assay target concentrations and the final DMSO concentration was equal or below 0.1%. Vero cells were pre-treated 4 hours prior to infection with either REM or MMPD diluted to the indicated concentration in DMEM, or a combination of the two drugs at the indicated concentrations diluted into DMEM (12-well plates, 1 mL per well). The media was removed at the time of infection, and the cells were inoculated with SARS-CoV-2 (MOI = 0.05) in 0.1 mL of inoculum, which was pre-mixed with the appropriate drug compound(s). The cells were incubated for 1 hour at 37°C to allow for infection. The cells were subsequently rinsed three times with 1 mL of DMEM, and then 1 mL of DMEM containing the appropriate drug compound(s) at the indicated concentration was added to each appropriate well. The cells were incubated for 0, 16, or 24 hours at 37°C before collecting a portion of each supernatant for titration. Upon collection, an equal amount of DMEM containing the compound was added back to each well. The infectious titer of SARS-CoV-2 for each sample was determined by TCID₅₀ assay in Vero cells. The collected samples were serially diluted ten-fold a total of six times in DMEM, with dilutions performed in quadruplicate. The resulting dilutions were used to inoculate Vero cells in 96-well plates, and the plates were allowed to incubate for 4 days at 37°C. Cells were then fixed by addition of 10% formalin to each well for 30 minutes, and subsequently stained for 5 minutes with crystal violet solution. TCID₅₀ values were obtained by counting the lowest dilutions in which infection was visible after crystal violet stain for two of four replicates. Each experiment was performed in biological triplicate, and the results were analyzed using a one-tailed t-test conducted in the GraphPad Prism 7 built-in statistical analysis software.

Results

We have previously observed a peak in SARS-CoV-2 titer at 24 hours post-infection, which plateaus through 48 hours post-infection¹¹. During the exponential growth phase, SARS-CoV-2 replication can be inhibited by 3-log with an overnight pre-treatment of MMPD at a concentration of 10µM, or 1.5-log with 4 hour pre-treatment at 5µM¹⁰.

Both REM and MMPD exhibit a potent antiviral effect on SARS-CoV-2 at concentrations of 2.5µM and 5µM after a 4 hour pre-treatment¹². REM pre-treatment at a 5µM concentration provides a 2.1-log decrease in titer at 16 hours-post infection (p=0.001) and a 3.9-log decrease in titer at 24 hours post-infection (p<0.001). MMPD causes a similar antiviral effect as REM at

the 5 μ M concentration, exhibiting a 2.1-log decrease in titer at 16 hours post-infection ($p=0.001$) and a 2.7-log decrease in titer at 24 hours post-infection ($p<0.001$). The effect of REM was dose-dependent, with the 2.5 μ M concentration of REM decreasing the titer by 1-log at 16 hours post-infection ($p=0.103$) and 1.5-log at 24 hours post-infection ($p=0.002$). However, MMPD antiviral effects appeared to be less dose-dependent, maintaining a 1.9-log decrease in titer at 16 hours post-infection ($p=0.003$) and a 2.5-log decrease in titer at 24 hours post-infection ($p=0.001$) at a concentration of 2.5 μ M (Figure 1). At sixteen hours post-infection 2.5 μ M REM does not have a statistically significant ($p<0.05$) difference between REM and untreated samples, whereas MMPD significantly reduced the infectious titer at this time point. When compared directly, MMPD causes a significantly greater reduction in SARS-CoV-2 infectious particles at both the 16-hour ($p=0.022$) and 24-hour ($p=0.014$) than REM. Therefore, MMPD provides a stronger inhibitory effect than REM at the 2.5 μ M concentration.

Since REM and MMPD inhibit virus replication via separate mechanisms, we tested the inhibitory effect of both drugs in combination. We tested combinations ranging from 5 μ M + 5 μ M to 0.31 μ M + 0.31 μ M (Figure 2), performing the same 4 hour drug pre-treatment. Inhibition of SARS-CoV-2 was dose-dependent, and the virus titer was reduced to undetectable levels at all time points ($p<0.001$) for concentrations of 1.25 μ M + 2.5 μ M, 2.5 μ M + 1.25 μ M, 2.5 μ M + 2.5 μ M, 2.5 μ M + 5 μ M, 5 μ M + 2.5 μ M, and 5 μ M + 5 μ M concentrations of REM and MMPD, respectively (Figure 2A, 2B). The lowest concentrations tested were 0.31 μ M REM + 0.31 μ M MMPD, which reduced the titer by 0.2-log at 16 hours post-infection ($p=0.371$) and 1.3-log at 24 hours post-infection ($p=0.015$). All combinations of concentrations between 0.31 μ M + 0.31 μ M and 1.25 μ M + 1.25 μ M produced a dose-dependent reduction in titer between 0.2-log and 2.5-log at 16 hours post-infection

and a dose-dependent reduction between 1.3-log and 3.7-log at 24 hours post-infection (Figure 2C).

We noticed a more robust inhibitory effect when the MMPD was provided at a lower concentration as opposed to REM being provided at a lower concentration ($p<0.001$, $p=0.01$), as illustrated 24 hours post-infection with the 0.31 μ M REM + 0.62 μ M MMPD and 0.62 μ M REM + 1.25 μ M MMPD to the 0.62 μ M REM + 0.31 μ M MMPD and 1.25 μ M REM + 0.62 μ M MMPD combinations (Figure 2C). This further supports that MMPD provides more potent SARS-CoV-2 inhibition than REM within the tested concentration ranges. More importantly, the data provides evidence that a treatment of REM + MMPD at a concentration as low as 2.5 μ M + 1.25 μ M reduces the production of infectious SARS-CoV-2 progeny to undetectable levels in cell culture.

Discussion

Our results provide evidence that MMPD potentially inhibits SARS-CoV-2 replication *in vitro* at concentrations as low as 2.5 μ M, which was the lowest concentration tested. Further work will determine the EC_{50} and EC_{90} for MMPD. Previous work established that cellular cytotoxicity for MMPD is greater than 10 μ M so the activity against the virus cannot be attributed to the drug being cytotoxic.

The inhibitory effect was similar for both MMPD and REM at 2.5 μ M at 24 hours post-infection. However, MMPD was the only drug to significantly inhibit SARS-CoV-2 at the 16 hour time point using a 2.5 μ M concentration. REM also appears to have a slightly higher range of dose-dependent efficacy in the 1–10 μ M range than MMPD. Here, we show a 2.4-log difference in antiviral activity for REM between 2.5 μ M and 5 μ M concentrations at 24 hours post-infection and only a difference of 0.2-log for MMPD using the same conditions. Previously, we determined that only a 1.5-log difference

Figure 1. Antiviral effect of either remdesivir (REM) or merimepodib (MMPD) on SARS-CoV-2 replication. Vero cells were pre-treated with either 2.5 μ M or 5 μ M concentrations of either REM or MMPD 4 hours prior to infection with SARS-CoV-2. The media was removed, and the cells were infected with SARS-CoV-2 (MOI = 0.05) pre-mixed with each respective compound. The cells were washed three times with DMEM, and 1 mL of media containing the respective compound was added. Cells were incubated at 37°C, and samples were collected at 0, 16, and 24 hours post-infection for TCID₅₀ titration. The experiments were performed in triplicate, and the averages of the three TCID₅₀ experiments are shown. The whiskers represent the standard deviation between the three results for each data point.

Figure 2. Antiviral effect of remdesivir (REM) provided in combination with merimepodib (MMPD) on SARS-CoV-2 replication. Vero cells were pre-treated with combinations of REM and MMPD ranging from 0.31 μM to 5 μM 4 hours prior to SARS-CoV-2 infection. The media was removed, and the cells were infected with SARS-CoV-2 (MOI=0.05) pre-mixed with each respective compound combination. The cells were washed three times with DMEM, and 1 mL of media containing the appropriate compound mixture was added. Cells were incubated at 37°C, and samples were collected at 0, 16, and 24 hours post-infection for TCID₅₀ titration. **A)** Co-treatment with concentrations between 5 μM and 1.25 μM. **B)** Co-treatment with equimolar concentrations of REM and MMPD between 5 μM and 0.31 μM. **C)** Co-treatment with concentrations between 1.25 μM and 0.31 μM. The 24-hour post-infection time point is further visualized using a bar graph. The experiments were performed in triplicate, and the averages of the three TCID₅₀ experiments are shown. The whiskers represent the standard deviation between the three results for each data point.

exists in MMPD inhibitory effect between 10 μM and 3.3 μM concentrations¹⁰.

The concentration of MMPD used in this study is well below the clinically-achievable concentration based on human PK trials. Oral administration of 50 mg MMPD achieves a plasma concentration of around 2500 ng/mL (5.5 μM) which makes it highly attractive for antiviral therapy². Intravenous administration of a 10 mg/kg dose of REM results in blood concentrations of 10 μM in NHPs¹³. REM and MMPD affect the replication of RNA viruses through different mechanisms. REM is an adenosine analogue that causes a delayed chain termination of RNA synthesis upon being incorporated into viral RNA⁴; whereas MMPD is a selective IMPDH inhibitor that manipulates the nucleoside triphosphate levels within cells, acting indirectly as a host-directed therapy. Therefore, we also tested whether the combination treatment using both, direct antiviral REM and host targeted drug MMPD could possibly have synergistic anti-COVID-19 activity. Our data demonstrate that this combination can completely abolish production of infectious virus in our cell culture model. Neither agent by itself completely abrogates viral production. While we are now determining the dose response of MMPD in this model system, REM data demonstrates

that a combination of both drugs still gives significant reduction of viral productions with low concentrations of both agent.

Our study indicates that MMPD alone, and/or in combination with REM, could be a viable treatment option for COVID-19 based on the potent inhibitory effect in cells culture at an easily achievable dose in the clinical setting. The drug could be quickly tested in a clinical setting based on its clinical trial history, and might be a useful prophylactic treatment to prevent spread of SARS-CoV-2 to the lower respiratory system¹⁰.

Data availability

Harvard Dataverse: Remdesivir and Merimepodib co-treatment for SARS-CoV-2. <https://doi.org/10.7910/DVN/HE6ZYJ2>.

This project contains the following underlying data:

- Raw Data REM + MMPD co-treatment for SARS-CoV-2.xlsx (SARS-CoV-2 inhibition data for remdesivir and merimepodib co-treatment in Vero cells)

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

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Mahmoud Kandeel

¹ Department of Biomedical Sciences, King Faisal University, Al-Ahsa, Saudi Arabia

² Department of Pharmacology, College of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt

- Despite the presence of several reports about the antiviral activity of remdesivir and merimepodib, the manuscript can provide useful information in the fight against SARS CoV2.
- Despite the provided piece of experiments and results of this manuscript, we still unable to precisely know the potency of these two drugs. The description of results was using the two concentrations of the two drugs without further important details. The potency of the drugs must be presented in terms of IC50. In this regard, several compounds were proved to be effective on SARS CoV-2 replication in the low nanomolar range and could be at least several folds more potent than REM and MMPD. In this case, the potency of REM and MMPD can be related to other anti-SARS CoV-2 drugs.
- The relation of the two drugs combination must be discussed in terms of additive effect, synergism or antagonism. For this purpose software as CompuSyn or similar can be used to calculate the combination index, which will scientifically assess the nature of these two drugs combination effect.
- "Oral administration of 50mg MMPD achieves a plasma concentration of around 2500ng/mL (5.5 μ M) which makes it highly attractive for antiviral therapy" recheck this sentence
- For assay reproducibility, another cell type is required e,g, Calu-3 cells
- The raw data of crystal violet stain pictures and plaques count could be provided as supplementary file.
- The two drugs seems to act on late stages of virus replication, at least not in the attachment

and fusion steps, the 4-hr pretreatment needs clarification. The time of addition experiment must be provided during different hours before and after infection.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology and drug discovery

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 26 May 2020

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Luka Cicin-Sain

Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany

The article "The IMPDH inhibitor merimepodib provided in combination with the adenosine analogue remdesivir reduces SARS-CoV-2 replication to undetectable levels *in vitro*" is a timely study on the effects of remdesivir and merimepodib against SARS CoV-2. The anti SARS CoV-2 effects of Remdesivir is not new, and neither is the antiviral activity of merimepodib, but the effect of merimepodib against SARS CoV-2 has not been shown in other studies and is a new

observation, along with the observation on additive effects of remdesivir and merimepodib in combination. The authors demonstrate robust antiviral activity of the two substances in the low micromolar concentration and a robust antiviral activity when both substances are administered in combination at a concentration of 1,25 μ M.

The major shortcoming of the study is that all results were generated on VeroE6, a green monkey cell line that is not necessarily representative of the natural sites of infection. This issue is particularly important because merimepodib acts by inhibiting inosine-5'-monophosphate dehydrogenase (IMPDH), a cellular enzyme, and potential polymorphisms between this gene in non-human primates and humans may result in a different efficacy of the drug in clinical settings. Therefore, the study would be strengthened by expanding the assay to human epithelial cells, ideally of lung origin.

Another smaller shortcoming of the study is that all statistical analysis was performed by t-tests, but the data are for the most part much more complex and require ANOVA analysis, and due to the low n in the experimental groups should be performed by Kruskal-Wallis. This in itself is not a major point of contention in my eyes. Reproducibility in experimental settings trumps statistical analysis by definition, the results in this study are clear already by eyeballing them, and the core results have been reproduced in several experiments shown in the two figures. Nevertheless, if statistical significance is used as an argument to validate the results, then one should use statistical methods that fit the experimental design and the group sizes.

In sum, despite these shortcomings, this is an important set of data that brings us closer to the development of antiviral strategies against the COVID19 pandemics and a substantial contribution to the field.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virology and immunology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have

significant reservations, as outlined above.

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