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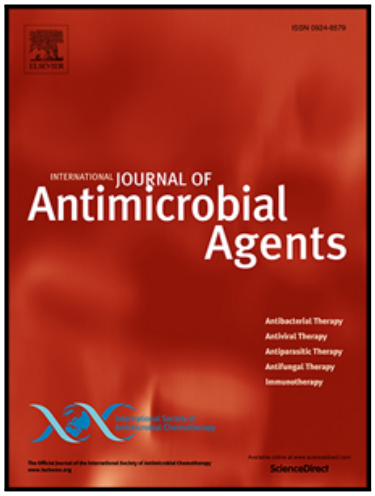


## Remdesivir-ivermectin combination displays synergistic interaction with improved in vitro activity against SARS-CoV-2

Laura N Jeffreys , Shaun H Pennington , Jack Duggan , Claire H Caygill , Rose C Lopeman , Alastair F Breen , Jessica B Jinks , Alison Ardrey , Samantha Donnellan , Edward I Patterson , Grant L Hughes , David W Hong , Paul M O'Neill , Ghaith Aljayyoussi , Andrew Owen , Stephen A Ward , Giancarlo A Biagini

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**FULL TITLE**

Remdesivir-ivermectin combination displays synergistic interaction with improved *in vitro* activity against SARS-CoV-2

**SHORT TITLE**

*In vitro* synergistic interaction of remdesivir and ivermectin against SARS-CoV-2

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**ABSTRACT**

A key element for the prevention and management of COVID-19 is the development of effective therapeutics. Drug combination strategies of repurposed drugs offer several advantages over monotherapies, including the potential to achieve greater efficacy, the potential to increase the therapeutic index of drugs and the potential to reduce the emergence of drug resistance. Here, we report on the *in vitro* synergistic interaction between two FDA approved drugs, remdesivir and ivermectin resulting in enhanced antiviral activity against SARS-CoV-2. Whilst the *in vitro* synergistic activity reported here does not support the clinical application of this combination treatment strategy, due to insufficient exposure of ivermectin *in vivo*, the data do warrant further investigation. Efforts to define the mechanisms underpinning the observed synergistic action, could lead to the development of novel therapeutic treatment strategies.

**KEYWORDS:** SARS-CoV-2, COVID-19, cytopathic activity, CPE, combination therapy, synergy

## INTRODUCTION

At the time of writing, the World Health Organisation (WHO) has reported more than 328 million cases of COVID-19, and more than 5.5 million deaths (1). There remains a clear need for therapeutic strategies with activity against SARS-CoV-2. Potential therapeutic strategies may include the repurposing of existing drugs as well as the discovery of novel therapies. Thousands of clinical trials are currently underway, with therapeutic approaches involving direct-acting antivirals, for the prevention of virus replication, and host-directed therapies aimed at mitigating against the disease pathology (2, 3).

Combination therapies can offer several advantages over monotherapies. They have the potential to achieve greater efficacy, to increase the therapeutic index of drugs and to reduce the emergence of drug resistance. Strategies to identify effective combination therapies are emerging, with several laboratories reporting *in vitro* combination screens (4) and *in vivo* animal combinations studies (5). In a recent clinical trial, baricitinib administered in combination with remdesivir was found to be superior, and to elicit fewer adverse effects, compared to either drug in isolation (6). Importantly, even in the absence of synergistic activity, an additive interaction between two drugs with separate mechanisms of action may profoundly reduce the speed at which drug resistance is established.

Both remdesivir and ivermectin have received attention for the treatment of COVID-19. Remdesivir is a prodrug C-adenosine nucleoside analogue that inhibits the viral RNA-dependent, RNA polymerase. Remdesivir was shown early in the pandemic to display *in vitro* antiviral efficacy against SARS-CoV-2 (7). In a double-blind, randomized, placebo-controlled trial, intravenous administration of remdesivir showed superiority relative to placebo in shortening the time to recovery in adults who were hospitalized with COVID-19 (8). However, other studies have suggested that its impact may be negligible (9), and on 20$^{th}$

November 2020 the WHO issued a conditional recommendation against the use of remdesivir in hospitalised patients (irrespective of disease severity) because there is no evidence supporting an improvement in survival or other outcomes in these patients.

Ivermectin is an anti-parasitic which is active against a wide range of parasites, including gastrointestinal roundworms, lungworms, mites, lice, hornflies and ticks (10). Ivermectin is reported to exhibit broad spectrum anti-viral activity against a wide range of RNA and DNA viruses (11). Recently, ivermectin was also shown to display anti-viral activity against SARS-CoV-2 (12), but approved doses are not expected to be high enough to achieve *in vitro*-defined target exposures systemically (13). Several clinical trials are now evaluating the potential of ivermectin for both prophylaxis and treatment of COVID-19, but the low exposures make the anti-inflammatory and/or immunomodulatory mechanisms of action more plausible than a direct antiviral activity of the monotherapy (14). In particular since studies with SARS-CoV-2 in Syrian Golden Hamsters showed an impact upon disease pathology in the absence of any effect on viral titres (15).

Here, using two distinct methodologies, determination of fractional inhibitory concentration index (FICI) with isobologram analyses, and checkerboard combination with SynergyFinder analyses, we report a synergistic interaction between remdesivir and ivermectin resulting in improved *in vitro* antiviral activity against SARS-CoV-2. The data are discussed in the context of current therapeutic efforts against COVID-19.

## MATERIALS AND METHODS

**SARS-CoV-2 Strain.** SARS-CoV-2/Human/Liverpool/REMRQ0001/2020 was isolated from a nasopharyngeal swab from a patient in Liverpool and passaged a further 4 times in VERO E6 cells. The mapped RNA sequence has previously been submitted to Genbank, accession number MW041156.

**VERO E6 cell culture and plate preparation.** VERO E6 cells were maintained in complete EMEM (EMEM supplemented with 10% heat-inactivated (HI) FBS [Gibco; 10500-064] and 1% penicillin/streptomycin [Gibco; 15140-122]) in T175 flasks (Thermo Fisher Scientific) at 37°C with 5% $CO_2$. Cells were seeded in resting EMEM at $1 \times 10^5$ cells/well in 96-well plates (Grenier Bio-one; 655090). Plates were then incubated for 20 hours at 37°C with 5% $CO_2$ to allow the cells to reach 100% confluence. The resting minimal medium was then removed, and the cells used for downstream applications.

**Concentration-response for remdesivir and ivermectin against SARS-Cov-2.** VERO E6 cells were treated in triplicate with either drug in minimal medium at 25.00 μM, 8.33 μM, 2.78 μM, 0.93 μM, 0.31 μM, 0.10 μM and 0.03 μM (DMSO maintained at 0.25%) or control media, as appropriate. The plates were then incubated at 37°C with 5% $CO_2$ for 2 hours. The minimal media containing the experimental compounds and the control media was then removed. 50 μL minimal media containing SARS-CoV-2 (MOI of 0.05), 100 μL 2× semi-solid media and then 50 μL minimal media containing experimental compounds and control media was added to each well, as appropriate. After 48 hours, 4% v/v paraformaldehyde was added to each well and the plate incubated for 1 hour at room temperature. The medium was removed and cells were stained with crystal violet. Cells were washed three times with water and cytopathic viral activity was determined by measuring absorbance of each well at 590 nm using a Varioskan LUX microplate reader (Thermo Fisher Scientific).

Automated data quality control and data analyses were performed. For quality controls, for the viral control, any well which had a log-transformed value that was 2 standard deviations above the mean of all log-transformed viral controls was excluded; Similarly, for the non-viral control, any well which had a log-transformed value that was 2 standard deviations below the mean of all log-transformed non-viral controls was excluded. If, for either control, 2 or more wells were excluded on this basis, the plate was voided, and no further analysis performed. Next, $Z'$ was calculated for each plate using the uninfected/untreated controls and infected/untreated according to equation 1.

$$Z' = 1 - \frac{3(\hat{\sigma}_n + \hat{\sigma}_v)}{|\hat{\mu}_n - \hat{\mu}_v|} \qquad \ldots Eq.\ 1$$

Where $\hat{\sigma}_n$ and $\hat{\sigma}_v$ represent the standard deviation of the non-viral and viral controls respectively, while $\hat{\mu}_n$ and $\hat{\mu}_v$ represent the corresponding means of these controls. Drug activity was expressed as a percentage of inhibition of viral growth relative to the uninfected/untreated control (100% inhibition of viral cytopathic activity) and the infected/untreated control (0% inhibition of viral cytopathic activity) on that plate. $EC_{50}$ and $EC_{90}$ were calculated for each compound that generated a robust, converged four-parameter fit according to equation 2.

$$E = \frac{E_{MAX} \cdot C^h}{EC_{50}^h + C^h} \qquad \ldots Eq.\ 2$$

Where $\boldsymbol{E}$ is the drug effect at any given concentration ($\boldsymbol{C}$), $\boldsymbol{E_{MAX}}$ is the maximal level of viral inhibition (0%-100%), $\boldsymbol{EC_{50}}$ is the concentration required to achieve half of this maximal inhibition while $\boldsymbol{h}$ represent the hill slope which describes the steepness of the concentration-effect relationship.

Compounds that did not achieve ≥50% viral inhibition were deemed inactive without fitting. Concentrations that were deemed toxic as evidenced by more than a 20% (approximately two standard deviations of all data) drop in absorbance with concentration increase coupled with evidenced toxicity in drug controls were excluded from fitting analysis.

**FICI for remdesivir-ivermectin combinations against SARS-CoV-2.** Following the assessment of the inhibitory effect ($EC_{50}$) of remdesivir and ivermectin monotherapy on the cytopathic viral activity of SARS-CoV-2, the FICI was determined using the isobologram method developed by Berenbaum (16), using data from three independent biological replicates. Drug stocks were created in DMSO to provide a stock sufficient to produce a top concentration of 25 μM for each biological replicate. Drugs were then combined to generate mixed ratios of 1:0, 0.8:0.2, 0.6:0.4, 0.4:0.6, 0.2:0.8 and 0:1.0. Fixed ratios were then diluted across an 8-point concentration range 1:2 (DMSO maintained at 1%) to generate concentration-response data for each ratio, as previously described. Ratio dilutions were performed in a single 2 mL deep-well plate, and added in parallel to three 96-well plates for each biological replicate. One additional plate which was not inoculated with virus was included to observe drug toxicity. Compound incubation and viral addition was performed as described above. Z' was calculated and quality control implemented as above. Interpretation of FICI (FICI<=0.5 = synergy; FICI>4.0 = antagonism; FICI>0.5-4 = no interaction) was based on guidance provided by the *Journal of Antimicrobial Chemotherapy* (17).

**Checkerboard combinations for remdesivir-ivermectin combinations against SARS-CoV-2.**

For robustness, a second method to assess pharmacodynamic drug combination interaction was utilised. Drug stocks were created by serial dilution. Compounds and controls were mixed 1:1 (DMSO maintained at 1%) to generate data for each combination alone and in

combination. Remdesivir was studied at 10 μM, 5 μM, 2.5 μM, 1.25 μM and 0.63 μM and ivermectin was studied at 5 μM, 2.5 μM, 1.25 μM, 0.63 μM and 0.31 μM. These concentrations were selected since they were determined not to cause cell toxicity to Vero E6 cells. Ratio dilutions were performed in a single 2 mL deep-well plate, and added in parallel to three 96-well plates for each biological replicate. Compound incubation and viral addition was performed as described above. $Z'$ was calculated and quality control implemented as above. Data was analysed using SynergyFinder and a summary synergy score generated (>10 is considered synergistic, −10 to +10 is considered additive, and <−10 is considered antagonistic) (18).

**RESULTS**

We assessed the capacity of remdesivr and ivermectin combinations to inhibit the in vitro cytopathic activity of SARS-CoV-2.

First, we determined the activity of each compound in isolation. For plates included in concentration-response analyses, the median signal to noise ratio was 29.3 and the median Z′ was 0.43 for concentration-response plates (Table 1). For each compound a robust 4-parameter fit was generated (Figure 1). For ivermectin the $EC_{50}$ was 2.4 ± 1.1 μM and for remdesivir the $EC_{50}$ was 1.3 ± 2.1 μM (geometric mean ± geometric standard deviation).

We subsequently determined the combination interaction between remdesivir and ivermectin by isobologram. The median signal to noise ratio was 26.4 and the median Z′ was 0.49 for concentration-response plates (Table 1). The 0.2:0.8 (remdesivir:ivermectin [5 μM:20 μM]) ratio 0.4:0.6 ratio (10 μM:15 μM) and 0.6:0.4 (15 μM:10 μM) ratio, demonstrated synergy (FICI<0.5) across all 3 biological replicates (Figure 2). For the 0.8:0.2 (:20 μM:5 μM) ratio just one biological replicate met the defined threshold hold of synergy (Figure 2). The other two biological replicates did, however, exceed the predicted effect assuming a purely additive relationship (Figure 2A).

We confirmed the synergistic interaction using interaction potency models using the SynergyFinder platform (18). The median signal to noise ratio was 23.6 and the median Z′ was 0.62 for concentration-response plates (Table 1). All four integrated synergy models determined that interactions between remdesivir and ivermectin were synergistic with synergy scores that far exceeded the threshold for synergy (Table 2 and Figure 2B).

**DISCUSSION**

Here, we described the synergistic interaction between two FDA approved drugs resulting in enhanced *in vitro* antiviral activity against SARS-CoV-2. Although combination therapy offers a number of advantages to monotherapy, genuine descriptions of synergy are relatively infrequent (19). Despite thousands of combination experiments having been performed, there have been very few reports of validated synergistic interactions against SARS-CoV-2 (4, 20).

At this stage, the mechanism underpinning the synergistic interaction between remdesivir and ivermectin is unclear; however, both drugs have previously been shown to inhibit SARS-CoV-2 replication (7, 12). Given that remdesivir is known to inhibit the RNA-dependent, RNA polymerase (21), it will be of interest to investigate whether ivermectin confers synergy by inhibiting an undefined alternative but complimentary role in RNA synthesis. Ivermectin has been shown to inhibit replication of HIV-1 and dengue through inhibition of importin β-mediated nuclear transport (22). In silico predictions suggest that ivermectin may interact with host-cell proteins such as importins, which are required for nuclear transport, as well as viral protein, including Nsp13 helicase and $M^{pro}$ protease, which facilitate replication and translation of SARS-CoV-2 (23). Further mechanistic studies will be required to determine the validity of these *in silico* predictions.

Special care was taken to assess *in vitro* activity across concentrations that likely cover the physiological exposure of remdesivir and invermectin in human plasma and lung tissue. In humans, a single 225 mg dose of remdesivir has been shown to produce a plasma $C_{MAX}$ of approximately 4,000 ng/mL (24), exceeding its *in vitro* $EC_{50}$ (1.3 ± 2.1 μM). In humans, a high dose of 600 μg/kg/day of ivermectin has been shown to produce a plasma $C_{MAX}$ of 120 ng/mL (25), much less than its *in vitro* $EC_{50}$ (2.4 ± 1.1 μM). The $C_{MAX}$ of remdesivir in lung epithelial lining fluid (ELF) has not been established and it is likely that these concentrations

are important with regards clinical activity. Poor exposure in lung ELF may well explain the limited impact of remdesivir in the clinic (8). Interestingly, concentrations of ivermectin are predicted to be some 3-fold higher in the lung than in plasma (26); however, even at these levels ivermectin fails to meet its *in vitro* $EC_{50}$ and no data are presented here, or elsewhere, that would support the clinical application of ivermectin for the treatment of SARS-CoV-2 infection. Given that 88-93.6 % of remdesivir (27) and 93.2 % of ivermectin (28) is protein bound, the availability of unbound drug at target sites is predicted to be considerably less than the reported values based on total drug concentrations.

Data presented here demonstrate that remdesivir administered in combination with ivermectin enhances *in vitro* antiviral activity. As described above, with respect to ivermectin, due to insufficient exposure of unbound drug at the target site, this combination strategy does not represent a clinically tractable therapeutic strategy. In addition, the differing routes of administration would likely impact the ability to achieve therapeutic concentrations of both drugs simultaneously. Further investigations are now required to determine whether the observed synergistic interaction can be replicated in animal disease models and with drugs that share similar modes of action, such as for example the orally bioavailable polymerase inhibitors, favipiravir or molnupiravir. The underpinning mechanisms for this synergy warrant further investigation so that this pharmacodynamic phenomenon can be exploited for the development of optimal drug combinations.

## DECLARATIONS

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**Competing Interests:** A.O. is a Director of Tandem Nano Ltd. A.O. has received research funding from ViiV, Merck, Janssen and consultancy from Gilead. These associations had no influence over the content of the current manuscript. P.O.N. is currently engaged in a collaboration with Romark LLC but this interaction had no influence over the content of the current manuscript. No other conflicts are declared by the authors.

**Ethical Approval:** Not required

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***Table 1.*** *Assay performance measures.*

| | **Concentration-response** | **Isobologram** | **Checkerboard** |
|---|---|---|---|
| **Total number of plates analysed** | 6 | 9 | 9 |
| **Signal to noise ratio (Median [Range])** | 29.3 (19.6 - 39.4) | 26.4 (13 - 37.3) | 23.6 (9.2 - 68.5) |
| **Signal to background ratio (Median [Range])** | 2.6 (1.9 - 4.1) | 1.9 (1.6 - 2.2) | 2.7 (2.3 - 3.5) |
| **Z′ (Median [Range])** | 0.43 (0.39 - 0.76) | 0.49 (0.18 - 0.7) | 0.62 (0.2 - 0.9) |

***Table 2.*** *SynergyFinder synergy score summary table for remdesivir and ivermectin.*

| | **Mean Synergy Score (Median [Range])** |
|---|---|
| **ZIP** | 35.33 (28.01 – 40.84) |
| **HSA** | 40.25 (36.02 – 44.34) |
| **Leowe** | 26.34 (26.04 – 30.45) |
| **Bliss** | 37.77 (27.61 – 41.69) |

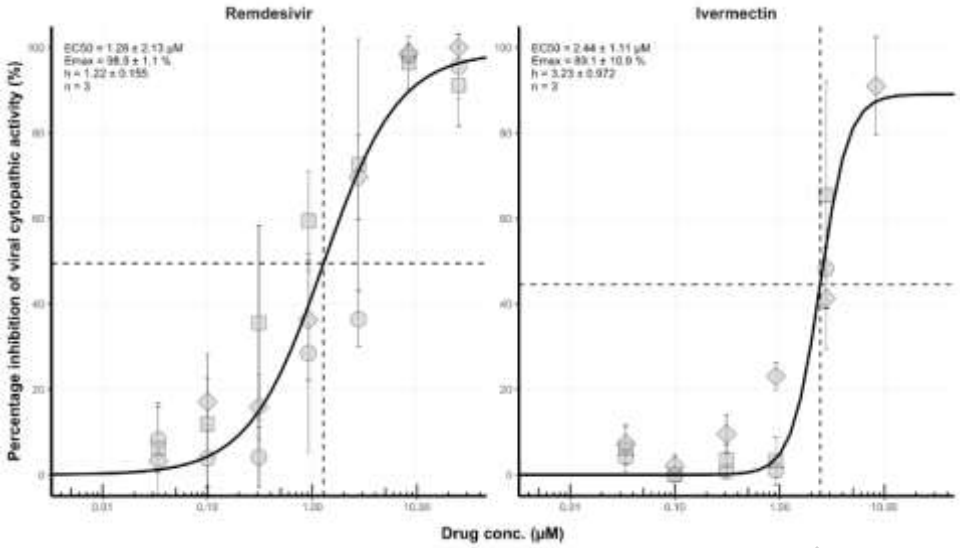


***Figure 1. Concentration-effect relationship for the inhibition (%) of SARS-CoV-2 cytopathic activity for remdesivir and ivermectin***. *For each compound, activity was expressed relative to uninfected/untreated controls (100% inhibition of viral cytopathic activity) and infected/untreated controls (0% inhibition of viral activity). For each compound, we assessed activity at 25.00 μM, 8.33 μM, 2.78 μM, 0.93 μM, 0.31 μM, 0.10 μM and 0.03 μM in triplicate. Data points impacted by drug toxicity were removed automatically. Non-linear regression using an $E_{MAX}$ model was performed on data taken from three independent biological replicates in order to generate concentration-effect predictions (solid black lines). For each compound, $EC_{50}$ values, hillslope and replicate number (n) are shown. Dashed lines represent the $EC_{50}$ of each compound. Squares, diamonds and circles represent individual biological replicates and error bars represent standard deviation calculated from technical triplicates .*

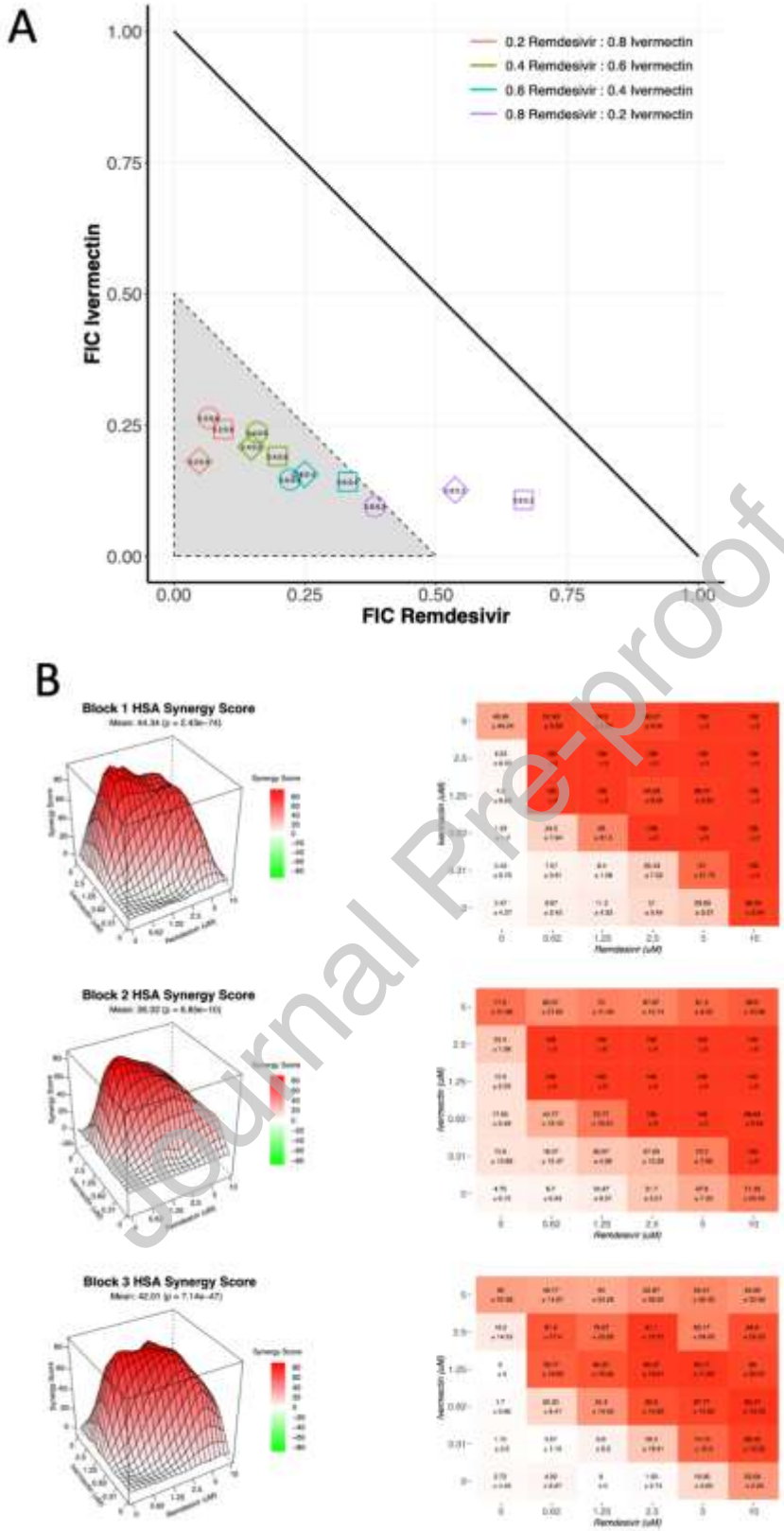


***Figure 2. Ivermectin and remdesivir display synergistic interaction.*** *(A) Using $EC_{50}$ values ranges of ivermectin and remdesivir were analysed for synergy from 7-point sigmoidal*

*curves. Data are presented for fixed concentrations at 25 µM (corresponding to 1.0), 20 µM (0.8), 15 µM (0.6), 10 µM (0.4) and 5 µM (0.2). The area indicating synergy (FIC <=0.5) is shown in grey. Squares, diamonds and circles represent individual biological replicates, each derived from technical triplicates. (B) 3D visualisation of compound integration based on the HAS synergy score (left) alongside heatmap showing compound combination dose-response matrices (right). 3D visualisations and matrices are shown for individual biological replicates, each derived from technical triplicates.*