

Supplementary Materials

Efficacy of Ion-Channel Inhibitors Amantadine, Memantine and Rimantadine for Treatment of SARS-CoV-2 in Vitro

Running title: Efficacy of Ion-Channel Inhibitors against SARS-CoV-2

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Supplementary Methods

Virus isolate. The corona virus isolate SARS-CoV-2/human/Denmark/DK-AHH1/2020 was isolated from a swap sample of a Danish patient by passage to VeroE6 cells. The used virus stock was derived after further passage in VeroE6 cells. The virus recovered from the previously described stock was sequence confirmed and had a SARS-CoV-2 infectivity titer of $5.5 \log_{10}$ TCID₅₀/mL [1].

Cells. African green monkey kidney VeroE6 cells [1] and human hepatoma Huh7.5 cells [2] were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Paisley, UK) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma, Saint Louis Missouri, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco/Invitrogen Corporation, Carlsbad, California, USA). Human lung carcinoma A549-hACE2 cells (Invivogen, Toulouse, France) were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Gibco, Paisley, UK) supplemented with 10% heat inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin as well as 0.5 µg/mL puromycin (Invivogen, Toulouse, France) [3]. Cells were maintained at 37°C and 5% CO₂ and split every 2-3 days with trypsin (Sigma, Saint Louis, Missouri, USA) to maintain a subconfluent cell layer.

Longer-term treatments of SARS-CoV-2 infected VeroE6 cell cultures. VeroE6 cells were seeded at 10^6 cells per flask in T25 cell culture flasks (Nunc). The following day cells were infected with SARS-CoV-2/human/Denmark/DK-AHH1/2020 at MOI 0.00002, and at the same time treated with the specified fold median effective concentrations (EC₅₀) of inhibitors. Subsequently, cells were split and treated with the specified concentrations of inhibitors upon cell splitting, every 2-3 days. Upon cell splitting, cell culture supernatants were obtained and stored at -80°C. The percentage of infected culture cells was evaluated by immunostaining for the SARS-CoV-2 spike protein and immunofluorescence imaging, as described below. Virus- or drug-induced

cytopathogenic effects were graded by inspection in the light microscope assigning four different grades (no, slight, pronounced and massive). When massive virus induced cell death occurred, cultures were closed following recovery of culture supernatant. Thus, for this final culture day supernatant but not immunostaining was available.

Evaluation of longer-term treatments by immunostaining. Procedures were previously described [3]. Upon cell splitting of longer-term treatment cultures and following addition of inhibitor, replicate cultures were seeded in chamber slides (Thermo Fisher Scientific, Rochester, NY, USA). The next day cells were fixed by submersion into methanol for 20min. Cells were then incubated with primary antibody SARS-CoV-2 spike chimeric monoclonal antibody (Sino Biological #40150-D004, Beijing, China) diluted 1:1,000 followed by incubation with secondary antibody Alexa-Fluor 488 goat anti-human IgG (H+L) (Invitrogen #A-11013, Paisley, UK) diluted 1:500 and Hoechst 33342 (Invitrogen, Paisley, UK) diluted 1:1,000. The % of infected cells was evaluated by fluorescence microscopy using a Zeiss Axiovert equipped with a 40× LD Plan-Neofluar objective (NA 0.6) (Jena, Germany), relating the estimated number of SARS-CoV-2 spike protein positive stained cells to the estimated number of Hoechst stained cells. Scores of 0% infected cells, single infected cells, 1%, 5% and 10%-90% infected cells in steps of 10% were assigned.

Next generation sequencing of SARS-CoV-2 genomes from supernatant in long-term treatment cultures. For extraction of SARS-CoV-2 RNA from cell culture supernatants as well as reverse transcription reaction (RT) and polymerase chain reaction (PCR) spanning the complete open reading frame by 5 overlapping amplicons, methodology was adapted from previously established methods for analysis of hepatitis C virus [1,4,5]. In brief, SARS-CoV-2 RNA was extracted from 100 µL cell culture supernatant with 300 µL Trizol LS (Life Technologies, Carlsbad, California, USA) and 100 µL chloroform (Sigma, Saint Louis, Missouri, USA) by use of 5PRIME

Phase Lock Gel Heavy tubes (Quantabio, Beverly, Massachusetts, USA). Then RNA was purified using RNA Clean and Concentrator™ – 5 (ZYMO, Irvine, California, USA), following the manufacturer's guidelines. Lastly, RNA was eluted in 30 µL nuclease-free H₂O (Ambion, Austin, Texas, USA). RT-PCR and PCR reagents and primers as well as thermocycler conditions were as described [1]. PCR amplicons were purified using DNA Clean and Concentrator™ – 25 (ZYMO, Irvine, California, USA) following the manufacturer's guidelines and eluted in 30 µL nuclease-free H₂O. Library preparations were done using NEBNext Ultra II DNA library Prep kit (New England BioLabs, Ipswich, Massachusetts, USA) and NGS analysis was done as described [1].

Short-term concentration-response treatments for determination of ion-channel inhibitor potency. Antiviral short-term concentration-response treatment assays were carried out as previously described [1,3,6]. VeroE6, Huh7.5 and A549-hACE2 cells were seeded in 96-well flat-bottom plates (Thermo Fischer Scientific, Roskilde, Denmark) at 10,000, 9,000 and 10,000 cells per well, respectively. The next day cells were inoculated with SARS-CoV-2/human/Denmark/DK-AHH1/2020 at MOI 0.002 for VeroE6 cells, MOI 0.02 for Huh7.5 cells, and MOI 0.003 for A549-hACE2 cells, based on the infectivity titer determined in VeroE6 cells. Following 1-hour incubation at 37°C and 5% CO₂ infected cells were treated with a dilution series of ion-channel inhibitors, or a dilution series of DMSO alone serving as a control for antiviral activity of DMSO. All treatment concentrations were tested in 7 replicates. Additionally, as all inhibitors were diluted in DMSO, inhibitor concentrations at which corresponding DMSO dilutions were expected to cause antiviral effects (residual infectivity < 70%) were excluded based on previously obtained results [3]. All treatment plates included 14 infected-nontreated wells serving as a positive control for infection, and 12 noninfected-nontreated blank wells serving as a negative control for infection. For A549-hACE2 cells, the concentration of DMSO was kept constant in all cultures. After 46-50 hours for

VeroE6 and A549-hACE2 cells, and after 70-74 hours for Huh7.5 cells, plates were subjected to immunostaining for the SARS-CoV-2 spike protein and evaluated as described below.

Short-term concentration-response combination treatments for analysis of interactions of ion-channel inhibitors with remdesivir. Interactions of ion-channel inhibitors and remdesivir for inhibition of SARS-CoV-2 were investigated as previously described [3]. The experimental setup was similar to that of short-term treatments with single ion-channel inhibitors described above. In brief, VeroE6 cells were seeded at 10,000 cells per well in 96-well flat-bottom plates. The next day cells were inoculated with SARS-CoV-2/human/Denmark/DK-AHH1/2020 at MOI 0.002.

Following 1-hour incubation at 37°C and 5% CO₂, infected cells were treated with a dilution series of inhibitors. Dilution series of ion-channel inhibitors singly, remdesivir singly or a combination of ion-channel inhibitors and remdesivir were chosen based on previously determined EC₅₀ values against SARS-CoV-2 in VeroE6 cells (see Figure 1 and Table 1 for ion-channel inhibitors as well as Gammeltoft and Zhou et al. for remdesivir [3]). Thus, for inhibitors and combinations of inhibitors infected cells were treated with a 1.5-fold dilution series with 10 different dilutions spanning the respective EC₅₀ values and aiming at achieving residual infectivities between 0 and 100 %. The same ion-channel inhibitor concentrations and remdesivir concentrations as used in single treatments were applied in the combination treatments; inhibitors were combined at a fixed ratio. All treatment conditions were tested in 7 replicates including 14 infected-nontreated replicates and 12 noninfected-nontreated replicates per experimental plate. After 46-50 hours incubation, plates were subjected to immunostaining for the SARS-CoV-2 spike protein and evaluated as described below.

Time-of-addition experiment with rimantadine. The time-of-addition experiment was carried out as previously described [6]. VeroE6 cells were seeded in 96-well flat-bottom plates (Thermo Fischer Scientific, Roskilde, Denmark) at 10,000 cells per well. The next day cells were inoculated

with SARS-CoV-2/human/Denmark/DK-AHH1/2020 at MOI 0.01 for a 2-hour infection phase. Following this infection phase, virus containing medium was removed and wells were washed one time with PBS, followed by addition of fresh growth medium. 230 μ M rimantadine was added at different timepoints post inoculation. For viral entry treatment, inhibitor was added together with the virus, at 0-hour post inoculation and removed together with the virus following the 2-hour infection phase. For post-entry treatment, inhibitor was added 2, 4 or 6 hours post inoculation and maintained throughout the experiment. Each treatment condition was tested in 6 replicates, and the experimental plate included 12 infected-nontreated cultures and 12 noninfected-nontreated cultures. After 46-50 hours the plate was subjected to immunostaining for the SARS-CoV-2 spike protein and evaluated as described below.

Immunostaining and evaluation of 96-well plates for short-term treatment assays with single inhibitors, and inhibitor combinations as well as for time-of-addition experiment. These procedures were carried out as previously described [1,3,6]. Cells were fixated and virus was inactivated by submersion into methanol (J.T.Baker, Gliwice, Poland) and incubation for 20 minutes at room temperature. For immunostaining of the SARS-CoV-2 spike protein, plates were first washed with PBS-tween [PBS (Sigma, Gillingham, UK) containing 0.1% Tween-20 (Sigma, Saint Louis, Missouri)] and blocked for endogenous peroxidase activity by addition of H₂O₂ and incubation for 10 minutes. This was followed by additional washing with PBS-tween and blocking by PBSK [PBS containing 1% bovine serum albumin (Roche, Mannheim, Germany) and 0.2% skim milk (Easis, Aarhus, Denmark)] for 30 minutes. Plates were then incubated with primary antibody SARS-CoV-2 spike chimeric monoclonal antibody (Sino Biological #40150-D004, Beijing, China) diluted 1:5,000 in PBSK for 2 hours at room temperature. Then plates were washed with PBS-tween and incubated with secondary antibody F(ab')₂-Goat anti-human IgG Fc Cross-Adsorbed Secondary Antibody, HRP (Invitrogen#A24476, Carlsbad, CA, USA) or Goat F(ab')₂ Anti-Human

IgG – Fc (HRP), preadsorbed (Abcamab#98595, Cambridge, UK), diluted 1:2,000 in PBSK. Lastly, plates were washed with PBS-tween, and SARS-CoV-2 spike protein positive cells were stained using DAB substrate BrightDAB kit (Immunologic # BS04-110, Duiven, Netherlands) according to the manufacturer's guidelines. Plates were evaluated by automated counting of single SARS-CoV-2 spike protein positive cells using an ImmunoSpot series 5 UV Analyzer (CTL Europe GmbH, Bonn, Germany). Mean of counts of infected-nontreated wells were 3,000-4,000 for VeroE6 cells, 1,000-2,000 for Huh7.5 cells and 2,000-3,000 for A549-hACE2 cells. The mean of counts from noninfected-nontreated wells was subtracted from counts of infected wells; this mean was typically lower than 50 counts. Counts from infected and treated wells were related to the mean count of the 14-replicate infected-nontreated wells to calculate % residual infectivity as:

$100 \times (\text{count in infected-treated culture} / \text{mean of counts in infected-nontreated cultures})$.

Datapoints are given as means of 7 replicates with standard error of the means (SEM). Sigmoidal concentration-response curves were fitted and EC50 values were calculated using Graphpad Prism 8.0.0 with a bottom constraint of 0 applying the formula $Y = \text{Top} / (1 + 10^{(\text{Log}_{10}\text{EC}_{50} - X) * \text{HillSlope}})$ [1,3,6]. Representative images from concentration-response antiviral treatment assays are shown in Zhou and Gilmore et al [6].

Interaction of ion-channel inhibitors with remdesivir was further investigated using the method of Chou and Talalay [7] and CompuSyn freeware (ComboSyn Inc) [8]. % inhibition values were calculated as (1-residual infectivity), which were designated fractional effect (Fa) values, ranging from 0.01 to 0.99, and which were entered into CompuSyn software. For each inhibitor or inhibitor combination 10 datapoints were entered based on 10 tested concentrations. The software was then used to determine (i) concentration-effect curves for single and combination treatments, (ii) combination index (CI) values and curves in relation to Fa values, and (iii) drug reduction index (DRI) values and curves in relation to Fa values. CI values were used to grade level of synergy and

antagonism as suggested by CompuSyn: $CI < 0.1$, very strong synergism; $0.1 \leq CI < 0.3$, strong synergism; $0.3 \leq CI < 0.7$, synergism; $0.7 \leq CI < 0.85$, moderate synergism; $0.85 \leq CI < 0.9$, slight synergism; $0.9 \leq CI < 1.1$, nearly additive; $1.1 \leq CI < 1.2$, slight antagonism; $1.2 \leq CI < 1.45$, moderate antagonism; $1.45 \leq CI < 3.3$, antagonism; $3.3 \leq CI < 10$, strong antagonism; $CI \geq 10$, very strong antagonism. DRI values describe how many folds the concentration of each inhibitor could be reduced due to synergism during combination treatment and at given levels of inhibition, compared to each inhibitor administered alone.

For evaluation of the time-of-addition experiment, counts from infected and treated wells were related to the mean count of the 12-replicate infected-nontreated wells to calculate % inhibition. % inhibition was calculated as $100\% - \% \text{ residual infectivity}$. Datapoints are given as means of 6 replicates with SEM. An image from the time-of-addition experiment is shown in Figure S7.

Cell viability concentration response assays. To evaluate cytotoxic effects of the inhibitors cell viability was monitored using a colorimetric assay, as previously described [3,6]. VeroE6, Huh7.5 and A549-hACE2 cells were seeded at 10,000, 9,000 and 10,000 cells per well, respectively, in 96-well flat-bottom plates. The following day cells were treated with dilution series of inhibitors. After 46-50 hours for VeroE6 and A549-hACE2 cells and after 70-74 hours for Huh7.5 cells, cell viability was evaluated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's guidelines. In brief, 20 μL CellTiter 96 Aqueous One Solution Reagent was added to each well and plates were then incubated for 1 to 3 hours at 37°C and 5% CO_2 . After incubation, for each well absorbance at 492 nm was recorded by use of a FLUOstar OPTIMA 96-well plate reader (BMG, LABTECH, Offenburg, Germany). Each inhibitor concentration was tested in 3 to 4 replicate wells and each experimental plate included 12 replicate nontreated control wells. Absorbance values of treated wells were related to the mean absorbance of the nontreated wells to estimate % cell viability. Datapoints are given as means of 3

to 4 replicates with SEM. Sigmoidal concentration-response curves were fitted and median cytotoxic concentration (CC50) values were calculated using GraphPad Prism 8.0.0 with a bottom constraint of 0 applying the formula $Y = \text{Top} / (1 + 10^{(\text{Log}_{10}\text{EC}_{50} - X) * \text{HillSlope}})$.

Supplementary Results

Amantadine, memantine and rimantadine interacted in a similar antagonistic manner with

remdesivir. To determine interactions of adamantane derivatives with remdesivir, the only approved drug for the treatment of COVID-19 directly targeting a SARS-CoV-2 protein, we carried out short-term combination treatment assays on 96-well plates.

Based on evaluation of concentration-response curves, for all tested ion-channel inhibitors the effect of the combination treatment did not exceed the effect of the single treatments, thus for example for amantadine at 73.2 μM and remdesivir at 1.7 μM , single treatments resulted in 80% and 60%, residual infectivity, respectively, while the combination treatment resulted in 59% residual infectivity (Figure 2 and Table S1).

Further analysis of the generated data using the software CompuSyn [8], based on a method developed by Chou and Thalalay [7], revealed mostly antagonistic interactions (Figure S5 and Table S2). Fa-CI plots suggested antagonism of ion-channel inhibitors with remdesivir at all key Fa values, with CI values being at least 1.6 (Figure S5 B, C and Table S2). In more detail, for amantadine + remdesivir, at key Fa values antagonism was suggested by CI values of 1.8. At Fa values of 0.02 to 0.99 at experimental datapoints mostly antagonism was suggested by CI values of 1.5 to 2.6. For memantine + remdesivir at key Fa values antagonism was suggested by CI values of 1.6 to 1.8 and at Fa values of 0.03 to 0.99 at experimental datapoints mostly antagonism was suggested by CI values of 1.5 to 2.7. For rimantadine + remdesivir at key Fa values antagonism was suggested by CI values of 1.6 to 1.9 and at Fa values of 0.01 to 0.99 at experimental datapoints mostly antagonism was suggested by CI values of 1.7 to 2.7. In agreement with the observed antagonism, combinations of ion-channel inhibitors with remdesivir suggested little to no drug reduction potential with most DRI values close to 1 (Figure S5 D and Table S2).

The combinations of inhibitors used in this assay did not cause cytotoxic effects (cell viability>90%) (Figure S6).

Supplementary Figures

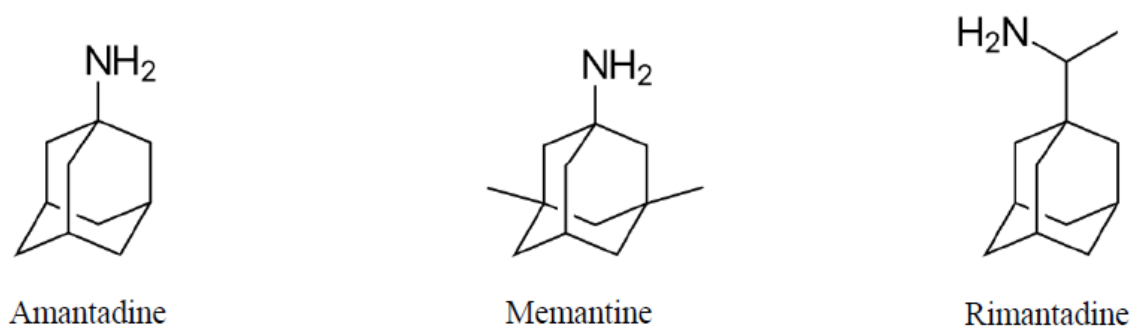


Figure S1. Structural formulas of ion-channel inhibitors investigated in this study

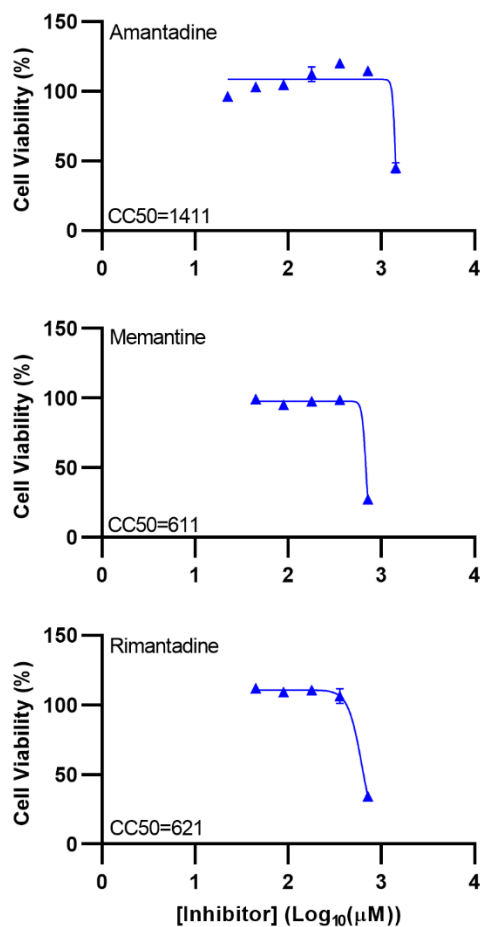


Figure S2. Cytotoxicity of ion-channel inhibitors in VeroE6 cells. Cell viability data were obtained in treatment assays in noninfected VeroE6 cells using a colorimetric assay. Data points represent % cell viability relative to the mean of 12 nontreated controls and are given as means of 3-4 replicates with SEM. Sigmoidal concentration response curves were fitted and CC50 values were determined using Graphpad Prism 8.0.0 applying the formula $Y = \text{Top} / (1 + 10^{(\text{Log}_{10}\text{EC}_{50} - X) * \text{HillSlope}})$ with a bottom constrain of 0. For all assays, additional data points on the lower plateau were excluded to improve curve fitting and determination of CC50 values; thus, only the lowest inhibitor concentration defining the lower plateau was included.

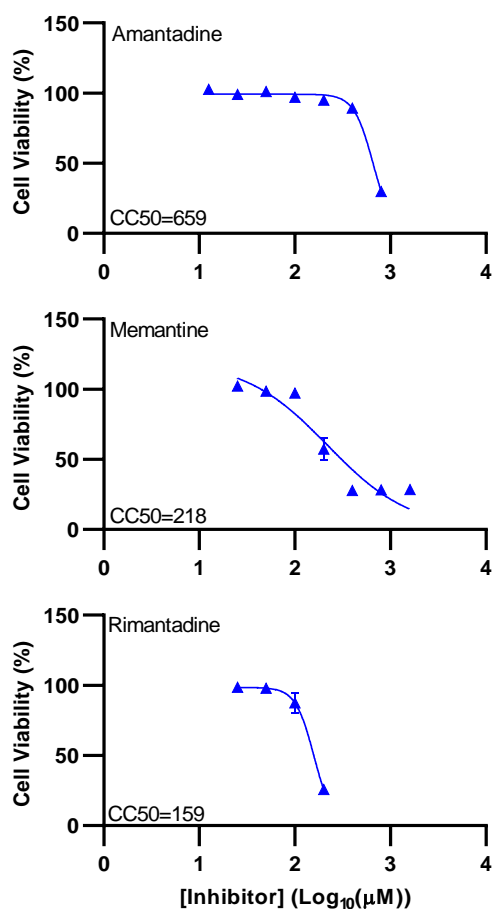


Figure S3. Cytotoxicity of ion-channel inhibitors in Huh7.5 cells. Cell viability data were obtained in treatment assays with noninfected Huh7.5 cells using a colorimetric assay. Data points represent % cell viability relative to the mean of 12 nontreated controls and are given as means of 3-4 replicates with SEM. Sigmoidal concentration response curves were fitted and CC50 values were determined using Graphpad Prism 8.0.0 applying the formula $Y = \text{Top} / (1 + 10^{(\text{Log}_{10}\text{EC}_{50} - X) * \text{HillSlope}})$ with a bottom constrain of 0. For all assays, additional data points on the lower plateau were excluded to improve curve fitting and determination of CC50 values; thus, only the lowest inhibitor concentration defining the lower plateau was included.

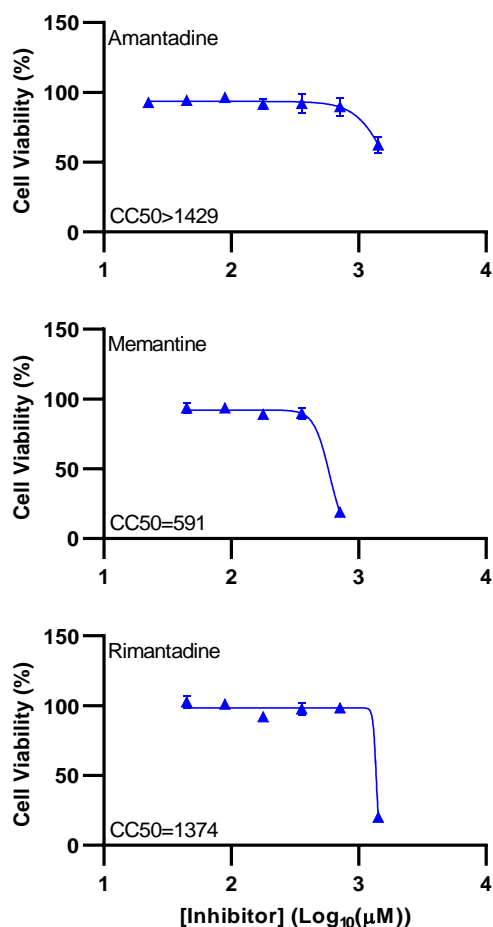


Figure S4. Cytotoxicity of ion-channel inhibitors in A549-hACE2 cells. Cell viability data were obtained in treatment assays with noninfected A549-hACE2 cells using a colorimetric assay. Data points represent % cell viability relative to the mean of 12 nontreated controls and are given as means of 3-4 replicates with SEM. Sigmoidal concentration response curves were fitted and CC₅₀ values were determined using Graphpad Prism 8.0.0 applying the formula $Y = \text{Top} / (1 + 10^{(\text{Log}_{10}\text{EC}_{50} - X) * \text{HillSlope}})$ with a bottom constrain of 0. For all assays, additional data points on the lower plateau were excluded to improve curve fitting and determination of CC₅₀ values; thus, only the lowest inhibitor concentration defining the lower plateau was included.

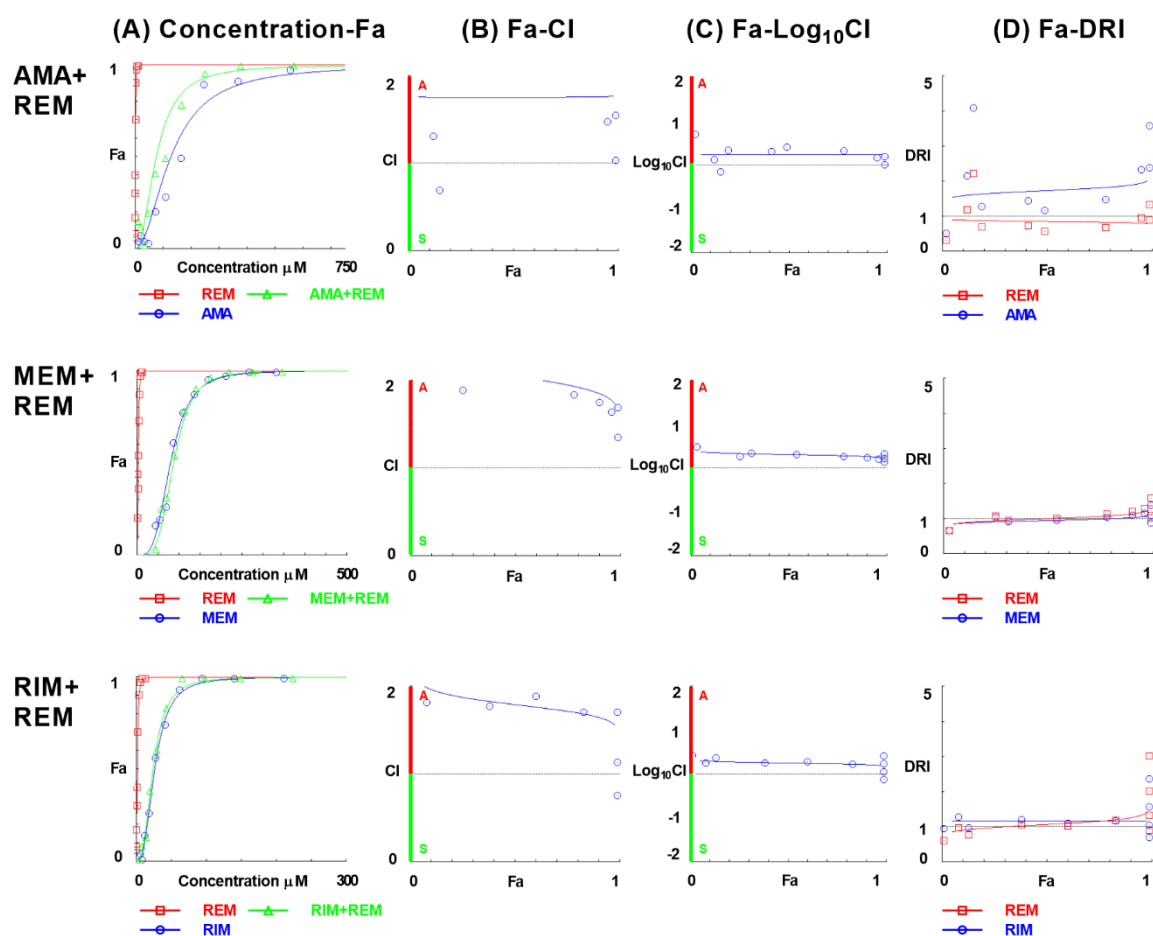


Figure S5. Analysis of interactions of ion-channel inhibitors with remdesivir.

VeroE6 cells seeded in 96 well plates were infected with SARS-CoV-2 and treated with specified concentrations of ion-channel inhibitors amantadine (AMA), memantine (MEM), rimantadine (RIM) or polymerase inhibitor remdesivir (REM) or a combination of ion-channel inhibitor and remdesivir. SARS-CoV-2 infected cells were visualized by immunostaining for SARS-CoV-2 spike protein and quantified by automated counting. Datapoints in panel A represent Fractional effect (Fa) values which were calculated by relating counts from infected and treated cultures to the mean count from infected-nontreated cultures, which were entered into CompuSyn software. Datapoints from representative experiments are given as means of 7 replicates and for each treatment 10 datapoints were entered. Combination index (CI) and drug reduction index (DRI) values were

calculated in CompuSyn based on the above described Fa values. For each inhibitor combination, the following curves were fitted using Compusyn: (A) concentration-Fa curves plotting Fa values ranging from 0.01 to 0.99 against specified inhibitor concentrations. (B) Fa-CI curves plotting CI values ranging from 0 to 2 against Fa values ranging from 0.01 to 0.99. (C) Fa-Log₁₀CI curves plotting logarithmic CI values ranging from 0.01 to 100 against Fa values ranging from 0.01 to 0.99. (B and C) Overall, CI values ≥ 1.1 suggest antagonism “A”, while CI values < 0.9 suggest synergism “S” indicated by green and red colour coding of the y-axis, respectively. (D) Fa-DRI curves plotting DRI values ranging from 0 to 5 against Fa values ranging from 0.01 to 0.99.

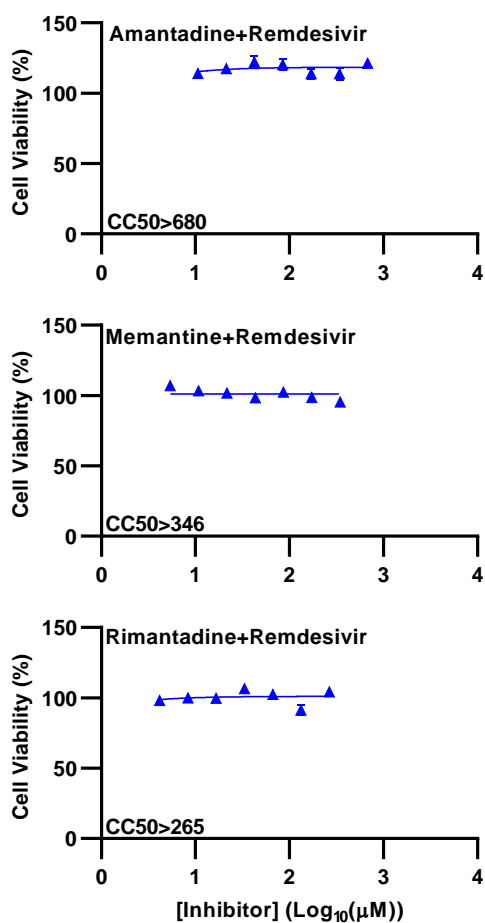


Figure S6. Cytotoxicity of ion-channel inhibitors in combination with remdesivir in VeroE6 cells. Cell viability data were obtained in treatment assays with noninfected VeroE6 cells using a colorimetric assay. Data points represent % cell viability relative to the mean of 12 nontreated controls and are given as means of 3-4 replicates with SEM. Concentrations are given as total concentration of inhibitor ([ion-channel inhibitor]+[remdesivir]). Sigmoidal concentration response curves were fitted and CC50 values were determined using Graphpad Prism 8.0.0 applying the formula $Y = \text{Top} / (1 + 10^{(\text{Log}_{10}\text{EC}_{50} - X) * \text{HillSlope}})$ with a bottom constrain of 0.

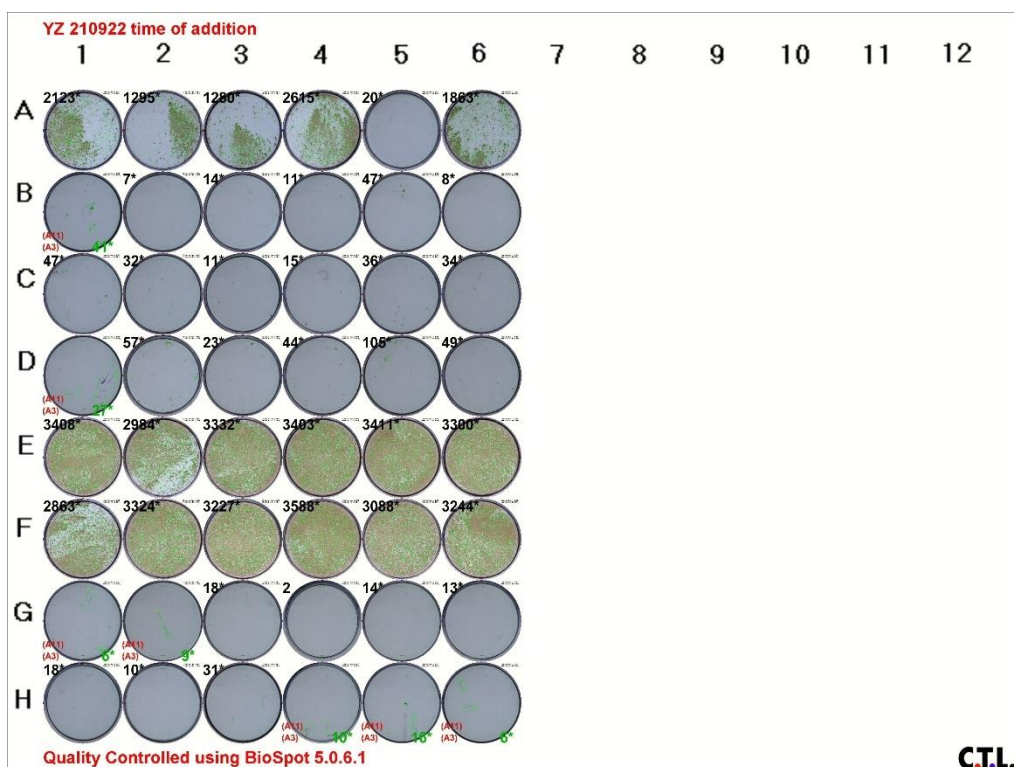
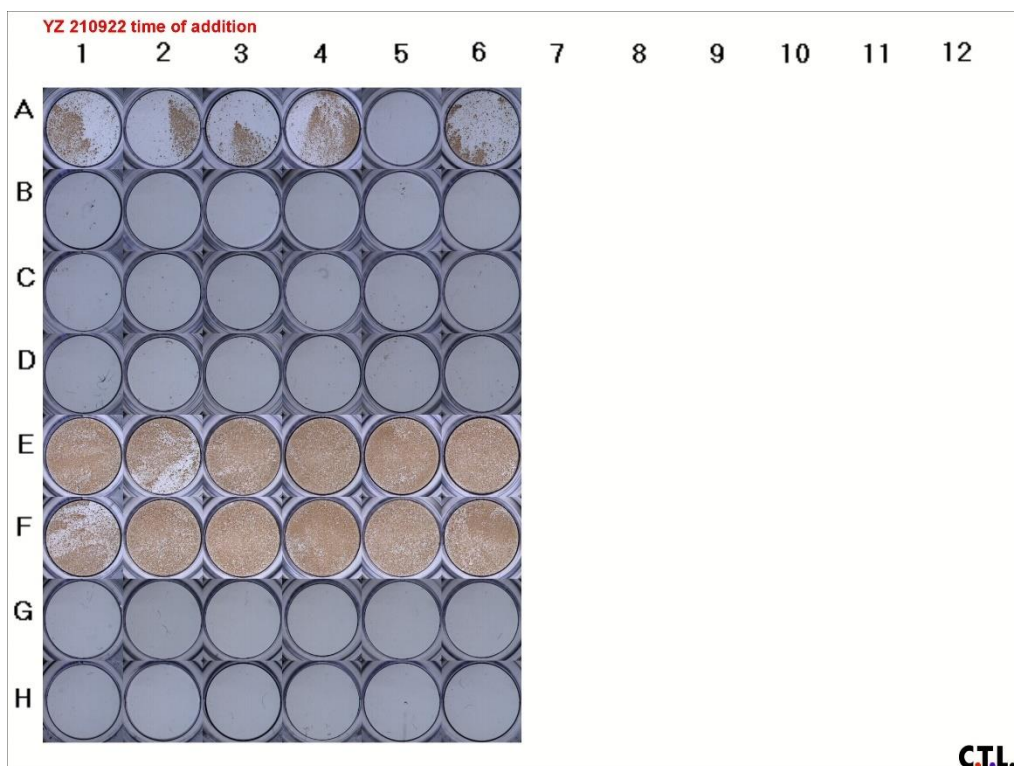


Figure S7. Image of 96-well plate from time-of-addition experiment in VeroE6 cells. The time-of-addition experiment was carried out in VeroE6 cells by inoculating with SARS-CoV-2 for a 2-hour infection phase and treating with rimantadine at different timepoints post viral inoculation. The

upper panel shows the experimental plate post staining, before automated counting. The lower panel shows the experimental plate post staining and post automated counting of SARS-CoV-2 infected cells. Each treatment condition was tested in 6 replicates and the experiment included 12 infected-nontreated and 12 noninfected-nontreated control cultures. The identity of cultures on the shown plate image is as follows: A1-A6: Rimantadine addition together with the virus (0 hour post inoculation) and rimantadine presence during the 2-hour viral infection phase; B1-B6: Rimantadine addition 2 hours post viral inoculation and presence throughout the experiment; C1-C6: Rimantadine addition 4 hours post viral inoculation and presence throughout the experiment; D1-D6: Rimantadine addition 6 hours post viral inoculation and presence throughout the experiment; E1-E6, F1-F6: Infected-nontreated control cultures; G1-G6, H1-H6: Noninfected-nontreated control cultures.

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