Hepatitis C Virus Protease Inhibitors Show Differential Efficacy and Interactions with Remdesivir for Treatment of SARS-CoV-2 in Vitro

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1 Hepatitis C Virus Protease Inhibitors Show Differential Efficacy and Interactions with
2 Remdesivir for Treatment of SARS-CoV-2 in Vitro

3 **Running title:** Efficacy of HCV Protease Inhibitors against SARS-CoV-2

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Abstract

Antivirals targeting SARS-CoV-2 could improve treatment of COVID-19. We evaluated efficacy of clinically relevant hepatitis C virus (HCV) NS3 protease inhibitors (PI) against SARS-CoV-2 and their interactions with remdesivir, the only direct-acting antiviral approved for COVID-19 treatment. HCV PI showed differential potency in short-term treatment assays based on detection of SARS-CoV-2 Spike protein in VeroE6 cells. Linear PI boceprevir, telaprevir and narlaprevir had 50% effective concentrations (EC50) of ~40 µM. Among macrocyclic PI, simeprevir had the highest (EC50 15 µM) and glecaprevir the lowest (EC50 >178 µM) potency, with paritaprevir, grazoprevir, voxilaprevir, vaniprevir, danoprevir and deldeprevir in between. Acyclic PI asunaprevir and faldaprevir had EC50 of 72 and 23 µM, respectively. ACH-806, inhibiting the HCV NS4A protease cofactor, had EC50 of 46 µM. Similar and slightly increased PI potencies were found in human hepatoma Huh7.5 cells and human lung carcinoma A549-hACE2 cells, respectively. Selectivity indexes based on antiviral and cell viability assays were highest for linear PI. In short-term treatments, combination of macrocyclic but not linear PI with remdesivir showed synergism in VeroE6 and A549-hACE2 cells. Longer-term treatment of infected VeroE6 and A549-hACE2 cells with 1-fold EC50 PI revealed minor differences in barrier to SARS-CoV-2 escape. Viral suppression was achieved with 3- to 8-fold EC50 boceprevir or 1-fold EC50 simeprevir or grazoprevir, but not boceprevir, in combination with 0.4- to 0.8-fold EC50 remdesivir; these concentrations did not lead to viral suppression in single treatments. This study could inform development and application of protease inhibitors for optimized antiviral treatments of COVID-19.

Keywords
coronavirus, antiviral, repurposing, combination treatment, synergy, COVID-19

Abbreviations
CC50, 50% cytotoxic concentration(s); CI, combination index(es); COVID-19, coronavirus disease
2019; CPE, cytopathogenic effect(s); DMSO, dimethyl sulfoxide; DRI, drug reduction index(es);
EC50, 50% effective concentration(s); Fa, fractional effect(s); FBS, fetal bovine serum; FDA, Food
and Drug Administration; hACE2, human angiotensin-converting enzyme 2; HCV, hepatitis C
virus; LLOQ, lower limit of quantification; Mpro, coronavirus main protease; NS, HCV
nonstructural protein; nsp, SARS-CoV-2 nonstructural protein; PBS, phosphate buffered saline;
PBSK, PBS containing 1% bovine serum albumin and 0.2% skim milk; PI, protease inhibitor(s);
PLpro, coronavirus papain-like protease; qPCR, quantitative polymerase chain reaction; RT, reverse
transcription; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SEM, standard error
of the mean(s); SI, selectivity index(es).
Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive-sense single-stranded RNA virus of the *Coronaviridae* family, which emerged in humans in 2019 most likely originating from a bat-borne virus.¹⁻³ SARS-CoV-2 causes coronavirus disease 2019 (COVID-19), a multi-systemic disease with initial symptoms mostly localizing to the respiratory tract. In the end of April 2021, the COVID-19 pandemic had been responsible for >152 million infected, >3 million deaths, and an unknown number of individuals suffering from long-term health effects.⁴⁻⁸ Repurposing of drugs approved for other medical indications is promoted as a time-saving approach to the identification of urgently needed treatments. At present, the only drug approved for treatment of COVID-19 that directly targets SARS-CoV-2 proteins is remdesivir, an inhibitor of the viral nonstructural protein (nsp) 12 polymerase, originally being an investigational broad-spectrum antiviral previously evaluated for treatment of chronic hepatitis C virus (HCV) infection and Ebola virus infection.⁹ Another important target of antiviral drugs are viral proteases, which are essential for the cleavage of viral polyproteins into functional proteins.¹⁰⁻¹³ The coronavirus main protease (M⁰⁰) or 3 chymotrypsin-like protease (3CL⁰⁰) is a cysteine protease corresponding to nsp5 and is highly conserved among coronaviruses. M⁰⁰ mediates 11 polyprotein cleavage events and is thus essential for viral replication.¹⁴⁻¹⁶ The second coronavirus protease, the papain-like protease (PL⁰⁰), is also a cysteine protease corresponding to the protease domain of nsp3 and is less conserved. PL⁰⁰ mediates 3 cleavage events and has important roles for viral replication and regulation of host innate immunity.¹⁷,¹⁸ Therefore, both SARS-CoV-2 proteases are considered potential drug targets.¹⁹,²⁰ Hepatitis C virus is a positive-sense single-stranded RNA virus of the *Flaviviridae* family, which was classified into 8 major genotypes and various subtypes.²¹,²² The main HCV protease,
nonstructural protein 3 (NS3), is a chymotrypsin-like serine protease.\textsuperscript{23–25} Together with its essential cofactor, NS4A, it mediates 4 cleavage events of the polyprotein. Inhibitors of this protease are important components of recently developed, highly efficient HCV treatment regimens based on the combination of antivirals directly targeting HCV proteins.\textsuperscript{26,27} Initially developed HCV protease inhibitors (PI) have a linear structure and include boceprevir and telaprevir, which were approved in 2011 in the US and EU, as well as narlaprevir, approved in 2016 in Russia, for treatment of chronic HCV infection (Supplemental Figure 1, Supplemental Table 1). Subsequently, PI with a macrocyclic structure, including simeprevir, paritaprevir, grazoprevir, glecaprevir, voxilaprevir, vaniprevir, danoprevir and deldeprevir were developed. These macrocyclic PI were approved between 2013 and 2019 in the US, EU or China, with the exception of vaniprevir, only approved in Japan, and deldeprevir, which was never approved. Of the 2 acyclic PI, asunaprevir and faldaprevir, asunaprevir was approved in Japan, Canada and China, while faldaprevir was not approved. Several of the initially developed PI were subsequently discontinued due to the development of more efficient PI with increased activity against the different HCV genotypes (Supplemental Table 1). At present, clinical use in the US, EU and China is focused on inhibitor combinations, including grazoprevir, glecaprevir or voxilaprevir. Additionally, in China, inhibitor combinations including paritaprevir, danoprevir or asunaprevir are used in the clinic. While an inhibitor of HCV NS4A (ACH-806) was tested in clinical phase 1 trials, development was halted due to reversible nephrotoxicity.\textsuperscript{28,29} In this study, we investigated \textit{in vitro} efficacy of a panel of HCV PI, including all clinically approved compounds and selected compounds tested in clinical studies, against SARS-CoV-2. These studies were carried out in African green monkey kidney VeroE6 cells and results were verified in human Huh7.5 hepatoma and A549 lung carcinoma cells, the latter engineered to constitutively express the human SARS-CoV-2 entry receptor angiotensin-converting enzyme 2.
(hACE2). All culture systems were previously demonstrated to be relevant for studies of antivirals targeting SARS-CoV-2.\textsuperscript{30–36} We further evaluated the efficacy of an HCV NS4A protease cofactor inhibitor. In concentration-response antiviral assays we determined 50\% effective concentrations (EC50), 50\% cytotoxic concentrations (CC50) and selectivity indexes (SI). Moreover, we evaluated interactions with remdesivir for selected linear and macrocyclic PI compounds. Finally, in longer-term cultures, we evaluated selected PI singly or in combination with remdesivir for their barrier to viral escape.
Results

Differential potency of clinically relevant HCV protease and cofactor inhibitors against SARS-CoV-2 in vitro. To determine the potency of a panel of HCV PI and an HCV NS4A inhibitor against SARS-CoV-2, we developed a cell-based short-term antiviral treatment assay in 96-well plates, adapting an assay previously developed to determine potency of HCV PI against HCV. In this assay, replicate SARS-CoV-2 infected cultures were treated with different dilutions of inhibitors. Following incubation, cultures were subjected to immunostaining for SARS-CoV-2 Spike protein and automated counting of single Spike protein positive cells. MOI and incubation time were chosen to avoid virus induced cytopathogenic effects (CPE) during the assay and to result in >1000 single Spike protein positive cells in nontreated control cultures, as determined in pilot CPE and immunostaining assays (for details see Materials and Methods). The potency of the inhibitors to reduce the number of Spike protein expressing cells compared to the nontreated controls was evaluated. Further, cytotoxicity of inhibitors was determined by cell viability assays; in these assays, cell viability >90% was confirmed for inhibitor concentrations used in treatment assays.

First, concentration-response studies were carried out in VeroE6 cells. Tested inhibitors were able to inhibit the virus with EC50 values in the micromolar range, with exception of glecaprevir, voxilaprevir and deldeprevir, where EC50 values could not be determined due to cytotoxicity of the drugs or antiviral activity of the diluent dimethyl sulfoxide (DMSO) at high drug concentrations (Figure 1, Table 1, Supplemental Figure 2). The linear PI boceprevir, telaprevir and narlaprevir showed comparable potencies with EC50 values of ~40 µM. Among the macrocyclic PI, simeprevir showed the highest potency with an EC50 of 15 µM. Further, paritaprevir had an EC50 of 22 µM, while grazoprevir and vaniprevir had EC50 values of 42 and 51 µM, respectively. Finally, EC50
was 87 µM for danoprevir. For the acyclic PI, faldaprevir (EC50 23 µM) was more potent than asunaprevir (EC50 72 µM).

To validate the immunostaining-based treatment assay, concentration-response assays quantifying CPE were carried out in VeroE6 cells for selected PI. In this assay, replicate SARS-CoV-2 infected cultures were treated with different dilutions of inhibitors. MOI and incubation time were chosen to induce relatively strong virus induced CPE in nontreated control cultures upon termination of the assay (30-50% cell viability). In treated cultures the potency of the inhibitors to inhibit virus induced CPE compared to the nontreated control cultures was evaluated. In these assays, the tested PI, boceprevir, simeprevir, grazoprevir, glecaprevir and voxilaprevir, showed similar potency and EC50 values as in the immunostaining-based assays (Supplemental Figure 3).

To confirm potency of PI in human cells, selected PI were studied in human Huh7.5 hepatoma cells. In these assays, the tested PI, boceprevir, simeprevir and grazoprevir, had similar concentration-response curves and EC50 values as in VeroE6 cells (Figure 2, Table 1).

Finally, we confirmed differential potency of selected PI in human A549 lung carcinoma cells transduced to express the SARS-CoV-2 entry receptor hACE2. In these A549-hACE2 cells, inhibitors showed slightly increased potency. Thus, boceprevir, simeprevir and grazoprevir showed an ~2-fold decreased EC50 compared to EC50 in VeroE6 cells. In line with this observation and in contrast to VeroE6 cells, the EC50 value for voxilaprevir could be determined in A549-hACE2 cells (10 µM). Similar to VeroE6 cells, no EC50 could be determined for glecaprevir in A549-hACE2 cells due to antiviral activity of DMSO at high inhibitor concentrations (Figure 3).

All inhibitors were diluted in DMSO. At the DMSO dilutions used for generation of specific data, no antiviral effect was observed in VeroE6, Huh7.5 or A549-hACE2 cells (Figure 1, 2 and 3 and Supplemental Figure 2).

Cell viability assays were carried out for all studied drugs to determine their level of in vitro...
cytotoxicity and CC50 values. In these assays drug concentrations were used at which no DMSO
induced cytotoxicity was observed (Figure 1, 2 and 3 and Supplemental Figure 2). In VeroE6 cells,
the linear PI showed the lowest cytotoxicity with all CC50 values above 200 µM (>1214, >432 and
269 µM for boceprevir, telaprevir and narlaprevir, respectively) (Figure 1, Table 1, Supplemental
Figure 4). Among the macrocyclic inhibitors, grazoprevir, glecaprevir and danoprevir showed the
lowest cytotoxicity with CC50 above 200 µM. Paritaprevir and vaniprevir showed intermediate
cytotoxicity with CC50 between 100 and 200 µM, while simeprevir, voxilaprevir and deldeprevir
showed the highest cytotoxicity with CC50 between 50 and 100 µM. Cell viability assays carried
out in Huh7.5 and in A549-hACE2 cells for selected PI showed similar results (Figure 2 and 3,
Table 1, Supplemental Figure 5 and 6).

Based on these assays, in VeroE6 cells the linear inhibitors had the highest selectivity indexes
(SI=CC50/EC50), >27.6 for boceprevir, >10.8 for telaprevir and 7.3 for narlaprevir (Table 1). Of
the macrocyclic inhibitors, paritaprevir and grazoprevir had the highest SI (5.6 and 5.7,
respectively), while simeprevir and vaniprevir had slightly lower SI of 3.9 and 3.4, respectively. For
glecaprevir, voxilaprevir, danoprevir, and deldeprevir SI could not be determined. For the acyclic
inhibitors, SI values were 6.3 for faldaprevir and 3.7 for asunaprevir. Finally, SI values calculated
based on assays in Huh7.5 and in A549-hACE2 cells were comparable to those based on assays in
VeroE6 cells (Table 1).

For the HCV NS4A inhibitor ACH-806, in VeroE6 cells EC50 was 46 µM, CC50 was >429 µM
and SI was >9.3 (Figure 1, Table 1, Supplemental Figure 4).

HCV PI showed differential interactions with remdesivir in short-term treatment assays. To
study interactions between selected PI and remdesivir, 96-well based short-term immunostaining-
based drug interaction assays were carried out. SARS-CoV-2 infected VeroE6 or A549-hACE2 cell
cultures were treated with selected PI singly, with remdesivir singly, or with a combination of PI
Inhibitor dilution series were chosen based on determined EC50 values (Table 1, Supplemental Figure 7). Inhibitors were only used at concentrations where no cytotoxicity or antiviral effects of DMSO were observed (Supplemental Figure 2, 8 and 9). For each inhibitor pair to be evaluated 7-10 treatment conditions were chosen. Each treatment condition was defined by a given concentration of PI applied singly, a given concentration of remdesivir applied singly, and a combination of these same concentrations of PI and remdesivir (Supplemental Table 2).

For each treatment condition, the residual infectivity of the culture receiving combination treatment was compared to the residual infectivity of the cultures receiving the corresponding single treatments. A graphical representation of the data is shown in Figure 4. All treatment conditions are detailed, and the corresponding residual infectivity values are shown in Supplemental Table 2.

For the linear inhibitors boceprevir and narlaprevir, at almost all treatment conditions the effect of the combination did not exceed the effect of the most efficient single inhibitor, remdesivir (Figure 4). For example, at treatment condition 3, 17.4 µM boceprevir resulted in 79% residual infectivity and 1.7 µM remdesivir resulted in 47% residual infectivity. Combination treatment with 17.4 µM boceprevir and 1.7 µM remdesivir resulted in 74% residual infectivity. Thus, treatment with remdesivir alone proved equally or more efficient than a combination of linear PI with remdesivir.

In contrast, for the macrocyclic inhibitors simeprevir, paritaprevir and grazoprevir, at various treatment conditions the effect of the combination exceeded the effect of both single inhibitors (Figure 4). For example, at treatment condition 3, 11.1 µM simeprevir resulted in 100% residual infectivity and 0.6 µM remdesivir resulted in 83% residual infectivity. However, combination treatment with 11.1 µM simeprevir and 0.6 µM remdesivir resulted in 1% residual infectivity. Thus, treatment with the combination showed an added effect compared to the corresponding single drug treatments.
We confirmed the above described differential interactions of linear versus macrocyclic PI with remdesivir in human A549-hACE2 cells by carrying out similar assays with the selected PI boceprevir, simeprevir and grazoprevir (Figure 5 and Supplemental Table 2).

To further define the nature of the interactions between the tested PI and remdesivir, the above-described datasets were analyzed using the method of Chou and Talalay using the Compusyn software. This analysis revealed primarily antagonistic interactions between remdesivir and the linear PI boceprevir and narlaprevir. In contrast, primarily synergistic interactions were observed between remdesivir and the macrocyclic PI paritaprevir and grazoprevir (Supplemental Text, Supplemental Figure 10 and 11, and Supplemental Table 3 and 4).

Compusyn software provided a suboptimal fit for the simeprevir+remdesivir datasets, most probably due to the steep slope of the simeprevir concentration-response curve. Therefore, an alternative software, SynergyFinder 2.0, was applied to analyze alternative datasets generated as required for this analysis and as described in the Supplemental Text. This analysis showed overall synergistic interactions between simeprevir and remdesivir in VeroE6 and A549-hACE2 cells (Supplemental Text and Supplemental Figure 12 and 13).

**HCV PI showed small differences in barrier to escape of SARS-CoV-2.** In order to investigate their barriers to escape, all PI, for which an EC50 could be determined, were used for longer-term treatment of SARS-CoV-2 infected VeroE6 cells in culture flasks at the highest possible equipotent concentration (1-fold EC50) according to predicted cytotoxicity (Figure 1, Table 1, Supplemental Figure 4). In the nontreated control cultures, the infection spread to 50% of culture cells on day 1 and to 90% of culture cells on day 3 post infection, as estimated by immunostaining for the SARS-CoV-2 Spike protein (Figure 6). Following day 3, typically massive virus induced cell death was observed in these control cultures. For all PI treated cultures (Figure 6), initial viral suppression was observed with 10-30% infected culture cells on day 1 post infection and treatment initiation. On day
3, only narlaprevir, grazoprevir, vaniprevir, asunaprevir and faldaprevir treated cultures showed viral suppression with infection of 10-50% of culture cells, while in boceprevir, telaprevir, simeprevir, paritaprevir and danoprevir treated cultures 90% of culture cells were infected. On day 5, virus spread to 90% of culture cells in grazoprevir treated cultures, while cultures treated with vaniprevir and asunaprevir were closed due to massive cell death, assumed to be due to PI induced cytotoxicity, possibly enhanced by SARS-CoV-2 infection. On day 7, in narlaprevir and faldaprevir treated cultures 60% of culture cells were infected; these cultures were closed on day 9 due to massive cell death.

Viral spread kinetics monitored by immunostaining were confirmed by determination of SARS-CoV-2 RNA titers in cell culture supernatants using an RT-qPCR assay (Figure 6). In the nontreated control culture, the number of SARS-CoV-2 genome copies increased by 4 orders of magnitude from 5.5*10^6/mL on day 1 to 9.7*10^{10}/mL on day 3 post infection. Compared to these values, all treatments resulted in a small decrease in genome copies on day 1 and 3; however, none of the treatments was able to prevent viral spread as monitored by determination of viral RNA titers.

To confirm PI barrier to escape in human cells, similar longer-term treatments were carried out in A549-hACE2 cells. Compared to the nontreated culture with SARS-CoV-2 RNA titers of 3.4*10^{10} and 2.5*10^{10} genome copies/mL on day 3 and 5, respectively, treatment with 1-fold EC50 of boceprevir, simeprevir and grazoprevir had no to little effect on viral spread monitored by determination of viral RNA titers (Figure 7, left panel). While A549-hACE2 cultures were also followed by immunostaining, in contrast to VeroE6 cells, % infection was difficult to estimate following day 1 post infection when a higher % of culture cells had become infected.

In conclusion 1-fold EC50 of the tested PI did not suppress SARS-CoV-2 in vitro.
Boceprevir had the potential to completely suppress viral infection *in vitro*. As suboptimal viral suppression was observed under treatment with 1-fold EC50, we chose the PI with the highest SI to enable longer-term treatment at higher fold EC50 concentrations. VeroE6 cells infected with SARS-CoV-2 were treated with 1-, 1.5-, 2-, 2.5, 3- and 5-fold EC50 of boceprevir (Figure 8). Treatment with 1- and 1.5-fold EC50 of boceprevir only had a minor impact on viral spread on day 1 post infection and treatment initiation, while 90% of culture cells became infected on day 3, as observed for nontreated control cells. Also, in cultures treated with 2 and 2.5-fold EC50, 90% of culture cells became infected on day 5. In contrast, treatment with 3- and 5-fold EC50 resulted in sustained viral suppression with no evidence of infected cells in the culture treated with 3-fold EC50 from day 3 and in the culture treated with 5-fold EC50 from day 1 during a follow-up period of 9 and 17 days, respectively. In addition, from cultures treated with 3- and 5-fold EC50 on day 5 and day 3, respectively, replicate cultures receiving no treatment going forward were derived, which did not show any infected cells during a follow-up period of 10 days, suggesting that the infection was cured under these treatments.

Viral spread kinetics monitored by immunostainings were confirmed by determination of SARS-CoV-2 RNA titers in cell culture supernatants (Figure 8). In cultures treated with 1- to 2.5-fold EC50 boceprevir viral RNA titers increased during the experiment; however, compared to the nontreated control culture this increase was decelerated in a concentration-dependent manner and overall peak titers were lower. In contrast, in cultures treated with 3- and 5-fold EC50 boceprevir, viral RNA titers decreased to values around the lower limit of quantification (LLOQ) during the experiment (Figure 8). In follow-up derived replicate cultures receiving no treatment, viral RNA titers were around the LLOQ.

To further confirm elimination of SARS-CoV-2 from cultures treated with 3- and 5-fold EC50 boceprevir, we carried out a 96-well based infectivity assay monitoring presence of infectious virus.
in all cell culture supernatants from the derived replicate cultures by inoculation of VeroE6 cell indicator cultures. Derived replicate cultures were chosen for this analysis as they were not treated with antivirals, which might inhibit infection of indicator cultures. Using this assay, we did not find evidence of any SARS-CoV-2 infected cells in the indicator cultures.

To investigate the potential of boceprevir to suppress SARS-CoV-2 infection in human cells, SARS-CoV-2 infected A549-hACE2 cells were treated with 1-, 2-, 3-, 5- and 8-fold EC50 boceprevir (Figure 7, middle panel). Virus RNA titrations revealed that 1- to 5-fold EC50 boceprevir only had a minor impact on viral spread while 8-fold EC50 boceprevir resulted in viral suppression with viral titers decreasing to values around the LLOQ during an observation period of 13 days. In addition, from the culture treated with 8-fold EC50 on day 5, a replicate culture receiving no treatment was derived, from which viral RNA titers were around the LLOQ during a period of 7 days. Thus, 3- to 8-fold EC50 boceprevir were able to suppress SARS-CoV-2 in vitro.

**Simeprevir or grazoprevir in combination with remdesivir completely suppressed viral infection in vitro.** To further study and confirm the interactions of PI with remdesivir, three PI with apparent differential interactions with remdesivir were selected for longer-term treatment of SARS-CoV-2 infected cells. VeroE6 cells infected with SARS-CoV-2 were treated with the PI boceprevir, simeprevir or grazoprevir singly, remdesivir singly, or either PI in combination with remdesivir, using 1-fold EC50 PI and 0.4-fold EC50 remdesivir. Inhibitor concentrations were selected to confer suboptimal effects in single treatments in order to rule out viral suppression in these treatments. Equipotent concentrations of PI were used based on data shown in Figure 1 and 6, and Table 1. For remdesivir, potency was evaluated based on concentration-response curves obtained from data shown in Figure 4 and Supplemental Figure 7, and in addition based on pilot longer-term treatment assays. Treatment with remdesivir, boceprevir, simeprevir or grazoprevir singly, as well...
as treatment with boceprevir + remdesivir had none or only a minor impact on viral spread on day 1 post infection and treatment initiation, while 80 to 90% of culture cells became infected on day 3, as observed for nontreated control cells (Figure 9). In contrast, in cultures treated with simeprevir + remdesivir or grazoprevir + remdesivir complete and sustained viral suppression was achieved with no evidence of infection from day 1 during a follow-up period of 15 days. In addition, to confirm complete viral suppression, from these cultures replicate cultures receiving no treatment were derived on day 5. Replicate cultures derived from simeprevir + remdesivir and grazoprevir + remdesivir treated cultures did not show any infected cells during a follow-up period of 19 and 14 days, respectively.

Determination of SARS-CoV-2 RNA titers in cell culture supernatants confirmed viral spread kinetics monitored by immunostainings (Figure 9). Treatment with single inhibitors and boceprevir + remdesivir had no or a minor impact on RNA titers compared to the nontreated control culture (Figure 9). However, under treatment with simeprevir + remdesivir or with grazoprevir + remdesivir RNA titers decreased to values around the LLOQ during the experiment. In derived replicate cultures receiving no treatment, viral RNA titers were around the LLOQ.

Elimination of SARS-CoV-2 from cultures treated with simeprevir + remdesivir was confirmed by an infectivity assay monitoring the presence of infectious virus in all cell culture supernatants from the derived replicate cultures not receiving treatment.

To verify interactions of boceprevir, simeprevir or grazoprevir with remdesivir in human cells, SARS-CoV-2 infected A549-hACE2 cells were treated with 1-fold EC50 boceprevir, simeprevir or grazoprevir singly, 0.8-fold remdesivir singly, or either PI in combination with remdesivir (Figure 7, right panel). Virus RNA titrations revealed that treatment with single inhibitors or boceprevir + remdesivir only had minor impact on viral spread, while treatment with simeprevir + remdesivir or grazoprevir + remdesivir resulted in viral suppression with viral RNA titers around the LLOQ.
Thus, combination of simeprevir or grazoprevir with remdesivir was able to suppress SARS-CoV-2 \textit{in vitro}. 
Discussion

In the current study, we provided a head-to-head comparison of the efficacy of a panel of clinically relevant HCV PI, including all PI approved for treatment of chronic hepatitis C, against SARS-CoV-2 in cell-based assays. In short-term antiviral assays, PI showed differential potency with EC50 values between 15 µM (simeprevir) and >178 µM (glecaprevir) in VeroE6 cells and 9 µM (simeprevir) and >94 µM (glecaprevir) in A549-hACE2 cells. Detailed short-term synergy studies in both cell types using a PI sub-panel revealed PI structure dependent interactions with remdesivir, with linear inhibitors showing mostly antagonism and macrocyclic inhibitors showing mostly synergism. In longer-term VeroE6 and A549-hACE2 cell cultures, at relatively low equipotent concentrations PI showed small differences regarding barrier to escape. For boceprevir, a relatively high SI facilitated treatment with higher concentrations revealing its potential to completely suppress viral infection. Further, combination of simeprevir or grazoprevir with remdesivir suppressed viral infection at relatively low inhibitor concentrations, shown to be subtherapeutic in single treatments.

Even though cell lines do not entirely recapitulate *in vivo* conditions, they provide robust models for pre-clinical antiviral activity studies. Our findings are strengthened by the fact that similar results were obtained in three different cell lines, including two human cell lines of which one was derived from human lung. Future studies to evaluate the efficacy of HCV PI on SARS-CoV-2 in primary cells, organoids, animals or humans would be of interest.

The sequence homology between different SARS-CoV-2 isolates is high. In comparison to the Wuhan reference isolate (GenBank accession number NC_045512), the isolate used in this study harbored six consensus amino acid changes (T85I in nsp2, P323L in nsp12, E309K and D614G in S, Q57H in ORF3a, and R209I in N), however no changes in M\textsuperscript{pro} or PL\textsuperscript{pro}, the proposed main targets of the studied HCV PI.\textsuperscript{31}
EC50 against SARS-CoV-2 were in the micromolar range, with the lowest EC50 (9 and 15 µM for simeprevir in A549-hACE2 and VeroE6 cells, respectively) approaching EC50 of remdesivir (0.1 and 2.5 µM in A549-hACE2 and VeroE6 cells, respectively); EC50 of remdesivir were in line with previously reported results.\textsuperscript{30–32,35,36} However, EC50 of PI against SARS-CoV-2 were higher than EC50 against HCV: Initially developed HCV PI such as boceprevir and simeprevir were roughly 10- to 1,000-fold less potent, while optimized HCV PI such as grazoprevir, glecaprevir and voxilaprevir were roughly 1,000- to 100,000-fold less potent against SARS-CoV-2 than against different HCV isolates.\textsuperscript{29,38–40,42,47–49} This suggested that optimization of inhibition of the HCV protease counteracted broader activity against proteases of other RNA viruses, such as SARS-CoV-2.

Boceprevir showed the highest SI (>60.7 and >27.6 in A549-hACE2 and VeroE6 cells, respectively), while simeprevir showed one of the lowest SI (6.2 and 3.9 in A549-hACE2 and VeroE6 cells, respectively). Of note, some clinically relevant drugs such as digoxin have low therapeutic breadth with SI as low as 2,\textsuperscript{50} and HCV PI have proven safe in clinical practice. To estimate the clinical potential of inhibitors, comparison of their EC50 with clinically achievable plasma and tissue concentrations is more relevant than comparison with \textit{in vitro} CC50 values. For most HCV PI, peak plasma concentrations (Cmax) were significantly lower than the determined EC50 values (Supplemental Table 5). The most favorable Cmax/EC50 ratio was found for simeprevir (Cmax/EC50 of ~1), followed by faldaprevir (Cmax/EC50 of ~0.2), as well as boceprevir, telaprevir and vaniprevir (Cmax/EC50 of ~0.1) based on EC50 obtained in VeroE6 cells. Further, plasma concentrations of free and biologically active compounds are expected to be lower than reported Cmax due to the high plasma protein binding of HCV PI. Regarding tissue concentrations, for HCV PI mostly liver concentrations were reported and were 20- to 280-fold higher than plasma concentrations.\textsuperscript{43,51–53} In rats, following a single oral administration of
simeprevir, concentrations in the intestine, which is permissive to SARS-CoV-2 infection, were up to 128-fold higher than in the plasma, while concentrations in other tissues were roughly equal to plasma concentrations. Therefore, it would be relevant to determine HCV PI lung concentrations in humans following multiple doses in steady state. For remdesivir showing high in vitro efficacy clinical efficacy might in part be limited by poor distribution to the lungs. Poor lung distribution following systemic application of inhibitors might be overcome by improved formulations allowing topical application, such as inhalable formulations.

Inhibitor efficacy can be improved by combination treatments with synergistic and thus drug saving effects as reported here for combination of remdesivir with the macrocyclic PI simeprevir, paritaprevir or grazoprevir in VeroE6 cells and with simeprevir or grazoprevir in A549-hACE2 cells. Synergism was recently suggested for combination of remdesivir with simeprevir or grazoprevir in short-term assays in VeroE6 cells and human embryonic kidney (HEK293T) cells. Our extensive results in VeroE6 and A549-hACE2 cells using short-term drug interaction assays demonstrated that the mode of interaction between PI and remdesivir depended on the PI structure. Thus, combination of remdesivir with the linear PI boceprevir and narlaprevir showed mostly antagonism, while combination with macrocyclic PI showed mostly synergism. In contrast to the previous studies, we confirmed these PI structure dependent interactions with remdesivir in longer-term treatment assays in VeroE6 and A549-hACE2 cells, where combination of remdesivir with simeprevir or grazoprevir, but not with boceprevir, resulted in added efficacy. This structure dependence might be explained by differences in viral targets. While the investigated HCV PI were suggested to target MPro, simeprevir was suggested to also target the SARS-CoV-2 polymerase and simeprevir, grazoprevir and paritaprevir were suggested to also target PLpro. It should be noted that additional alternative viral targets, including nsp13 (helicase), nsp14 (exonuclease and methyltransferase), nsp15 (endoribonuclease), nsp16 (2'-o-
ribose methyltransferase), as well as structural proteins N (capsid) and Spike, were suggested for paritaprevir, grazoprevir and simeprevir by modelling studies.\textsuperscript{74,78–83} Future detailed molecular studies are required to fully define the viral targets of different HCV PI.

HCV PI were designed and optimized to bind the HCV NS3 protease. In modelling studies, structural similarity between the HCV NS3 protease and SARS-CoV-2 M\textsuperscript{pro}, including their active sites, was reported\textsuperscript{36,57,68,77}, despite a lack of overall sequence conservation. While both viral proteases are chymotrypsin like proteases, the HCV NS3 protease has a larger and more shallow binding groove.\textsuperscript{34} The HCV NS3 protease and PL\textsuperscript{pro} do not show structural similarity.\textsuperscript{36}

While carrying out and revising this study, several research articles addressing a potential effect of HCV PI on SARS-CoV-2 were published. Using \textit{in silico} modelling approaches, more than 20 studies predicted binding of different linear and macrocyclic HCV PI to SARS-CoV-2 M\textsuperscript{pro}.\textsuperscript{34,36,66–75,57,76,58–61,63–65} Fewer studies predicted binding of macrocyclic HCV PI to PL\textsuperscript{pro}.\textsuperscript{36,62} Further, crystal structures of boceprevir, narlaprevir and telaprevir bound to M\textsuperscript{pro} were solved.\textsuperscript{34,77,84–86} In addition, reports were published on inhibition of M\textsuperscript{pro} by linear and macrocyclic HCV PI \textsuperscript{33–36,84,85} and of PL\textsuperscript{pro} by macrocyclic and acyclic HCV PI.\textsuperscript{36}

Recently, five groups demonstrated efficacy of different HCV PI in VeroE6 cells.\textsuperscript{33–36,87} Most EC50 values reported in these studies were in the same range as those reported here; slightly higher EC50 values observed in our study are most likely caused by differences in experimental assay conditions. EC50 in human lung cells were only reported for simeprevir (EC50 1 \textmu M)\textsuperscript{15} and voxilaprevir (>10 \textmu M), also using the A549-hACE2 cell line.\textsuperscript{30} The similar PI EC50 values in monkey VeroE6 cells, human Huh7.5 and human A549-hACE2 cells reported in our study validate VeroE6 cells for the study of HCV PI efficacy against SARS-CoV-2. Further, the short-term treatment assay based on quantification of SARS-CoV-2 Spike protein expressing cells was validated here by an assay measuring virus induced CPE.
Finally, we report antiviral activity of the HCV NS4A inhibitor ACH-806. Future studies are required to define the SARS-CoV-2 target of this compound and to investigate its potential to inform design of SARS-CoV-2 inhibitors.

In conclusion, we here provide a head-to-head comparison of the efficacy of a panel of clinically relevant HCV PI against SARS-CoV-2, including detailed studies of interaction with remdesivir using different cell lines. Of currently clinically widely used HCV PI, grazoprevir and voxilaprevir showed activity against SARS-CoV-2 in lung cells. For clinical use of HCV PI with higher potency and higher plasma concentrations such as simeprevir, production would need to be re-initiated, as was the case for remdesivir. While HCV PI showed relatively low Cmax/EC50 ratios assuming systemic administration, it remains possible that active concentrations will be attainable in relevant tissues. Further, treatment efficacy might be increased by topic administration of improved formulations and by combination with remdesivir. As this study demonstrated structure dependent differential interaction of HCV PI with remdesivir, novel PI should be tested for interaction with remdesivir if combination treatment is considered. While clinical studies would be needed to investigate if HCV PI studied here could have direct clinical relevance, this study identifies compounds that could assist the development of further optimized PI molecules for future COVID-19 treatment regimes.
Materials and Methods.

Cell cultivation. All cells were maintained at 37°C and 5% CO₂. African green monkey kidney VeroE6 cells (gift from J. Dubuisson) and human Huh7.5 hepatoma cells 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) and 100 U/mL penicillin with 100 μL streptomycin (Gibco/Invitrogen corporation, Carlsbad, California, USA). 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 with 100 μL streptomycin and 0.5 μg/mL puromycin (Invivogen, Toulouse, France). Cells were split every 2-3 days with trypsin (Sigma, Saint Louis, Missouri, USA) to maintain a subconfluent monolayer.

Virus isolate. The corona virus isolate SARS-CoV-2/human/Denmark/DK-AHH1/2020 was derived from a swab sample from a Danish patient that was passaged in VeroE6 cells. For the experiments presented here we used a sequence confirmed 2nd viral passage stock with an infectivity titer of 5.5 log₁₀ TCID50/mL.

Inhibitors. All inhibitors were purchased from Acme Bioscience (Palo Alto, California, USA) and dissolved in DMSO (Sigma, Saint Louis, Missouri, USA).

Short-term concentration-response antiviral treatment assays for evaluation of inhibitor potency. 96-well-based short-term antiviral treatment assays in VeroE6 cells, Huh7.5 cells and A549-hACE2 cells were developed based on assays previously established for determination of the potency of HCV PI against HCV. VeroE6 and A549-hACE2 cells were seeded at 10,000 cells per well and Huh7.5 cells were seeded at 9,000 cells per well in 96-well flat-bottom plates (Thermo Fischer Scientific, Roskilde, Denmark). The following day, for VeroE6 cells medium was changed to 50 μL fresh medium and cells were inoculated with SARS-CoV-2/human/Denmark/DK-
AHH1/2020 at MOI 0.002 by adding 50 µL virus stock diluted in medium to each well. Huh7.5 cells were inoculated at MOI 0.02 and A549-hACE2 cells were inoculated at MOI 0.003 (based on the infectivity titer determined in VeroE6 cells) by exchanging the medium with 50 µL virus stock diluted in medium. Following 1-hour incubation at 37°C and 5% CO2, infected cells were treated with a dilution series of inhibitors by adding 50 µL medium with inhibitor resulting in the specified concentrations. Alternatively, cells were treated with a dilution series of DMSO alone serving as a control for antiviral activity of DMSO. All concentrations of inhibitor were tested in 7 replicates; 14 infected and nontreated and 12 noninfected and nontreated replicates were included in each assay.

For A549-hACE2 cells, the concentration of DMSO was kept constant in all cultures. Cells were subjected to immunostaining for the SARS-CoV-2 Spike protein and evaluated as described below after incubation for 46-50 hours for VeroE6 and A549-hACE2 cells or for 70-74 hours for Huh7.5 cells.

Additionally, 96-well based concentration-response CPE assays were carried out in VeroE6 cells. VeroE6 cells were seeded at 10,000 cells per well in 96-well flat-bottom plates (Thermo Fischer Scientific). The following day cells were inoculated with SARS-CoV-2/human/Denmark/DK-AHH1/2020 at MOI 0.01 by exchanging the medium with 50 µL virus stock diluted in medium. After 1-hour incubation, infected cells were treated with a dilution series of inhibitor by adding 50 µL medium with inhibitor resulting in the specified concentrations. All concentrations of inhibitor were tested in 4 replicates; 8 infected and nontreated and 16 noninfected and nontreated replicates were included in each assay. The concentration of DMSO was kept constant in all cultures. After 70-74 hours incubation at 37°C and 5% CO2, CPE was evaluated using Viral ToxGlo Assay (Promega, Madison, WI, USA) following the manufacturer’s guidelines. Relative light units (RLU) from infected and treated wells were related to the mean of RLU of the 16 noninfected control wells. Datapoints are given as means of 4 replicates with SEM. Sigmoidal concentration-response
curves were fitted and EC50 values were calculated using Graphpad Prism 8.0.0 applying the
formula $Y = \frac{\text{Top}}{1+10^\left(\log_{10}EC50 - X\right) \times \text{HillSlope}}$. 

**Immunostaining and evaluation of 96-well plates.** Cells were fixed and virus was inactivated by
submersion into methanol (J.T.Baker, Gliwice, Poland) for 20 minutes at room temperature. For
immunostaining for the SARS-CoV-2 Spike protein, plates were washed 2 times with PBS-tween
[PBS (Sigma, Gillingham, UK) containing 0.1% Tween-20 (Sigma, Saint Louis, Missouri)]. Then,
endogenous peroxidase activity was blocked by adding $\text{H}_2\text{O}_2$ and incubating for 10 minutes
followed by 2 more washes 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. Next, plates were emptied and 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 2 times with PBS-tween and
incubated for 1 hour at room temperature 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. Finally, plates were washed 2 times with PBS-tween, and SARS-CoV-2 Spike
protein positive cells were stained using DAB substrate BrightDAB kit (Immunologic # BS04-110,
Duiven, Netherlands) following 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). The mean of counts from noninfected and
nontreated wells, which was usually <50, was subtracted from counts of infected wells. Counts
from infected and treated wells were related to the mean count of the 14-replicate infected
nontreated wells to calculate % residual infectivity; mean counts of infected nontreated wells were
3,000-4,000 for VeroE6 cells, 1,000-2,000 for Huh7.5 cells and 2000-3000 for A549-hACE2 cells.
Datapoints are given as means of 7 replicates with SEM. Sigmoidal concentration-response curves were fitted and EC50 values calculated as described previously using Graphpad Prism 8.0.0 with a bottom constraint of 0 applying the formula \( Y = \frac{\text{Top}}{1+10^{\left(\log_{10}\text{EC50}-X\right)/\text{HillSlope}}} \). Representative images from concentration-response antiviral treatment assays are shown by Gilmore and Zhou et al.44

Short-term concentration-response antiviral treatment assays for analysis of interactions of PI and remdesivir in VeroE6 and A549-hACE2 cells. Interactions of selected PI in combination with remdesivir for inhibition of SARS-CoV-2 were investigated based on protocols previously established for HCV.38 The experimental design was similar to that of the concentration-response antiviral treatment assays described above. In brief, VeroE6 cells or A549-hACE2 cells were seeded at 10,000 cells per well in 96-well flat-bottom plates, medium was changed to 50 µL fresh medium, and cells were inoculated with SARS-CoV-2/human/Denmark/DK-AHH1/2020 at MOI 0.002 (VeroE6 cells) or MOI 0.003 (A549-hACE2 cells) by adding 50 µL virus stock diluted in medium to each well. Following 1-hour incubation at 37°C and 5% CO2, infected cells were treated with a dilution series of inhibitors by adding 50 µL medium with inhibitor resulting in the specified concentrations. Regarding inhibitor treatment, dilution series of selected PI singly, remdesivir singly or a combination of PI and remdesivir were used that were based on EC50 values against SARS-CoV-2. Thus, for inhibitors and combinations of inhibitors 1.15- to 2-fold dilution series with at least 7 different dilutions were applied spanning the respective EC50 values aiming at achieving residual infectivity between 0 and 100 %. For combination treatments the same PI and remdesivir concentrations as used in single treatments were applied with a fixed ratio, except for the simeprevir + remdesivir dataset where a non-constant ratio was used. All treatment conditions were tested in 6 or 7 replicates including 21 to 70 infected and nontreated replicates per experiment (with at least 7 replicates per experimental plate) and 12 noninfected and nontreated replicates per
experimental plate. In experiments with A549-hACE2 cells the concentration of DMSO was kept constant in all cultures. After 46-50 hours incubation, infected cells were visualized by immunostaining for the SARS-CoV-2 Spike protein and plates were evaluated by automated counting of single SARS-CoV-2 Spike protein positive cells, as described above.

**Concentration-response cell viability assays.** To evaluate cytotoxic effects of the inhibitors and DMSO, cell viability was monitored using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). VeroE6 cells, Huh7.5 and A549-hACE2 cells were seeded in 96-well flat-bottom plates at 10,000, 9,000 and 10,000 cells per well, respectively, and the following day treated with dilution series of inhibitors or combinations of inhibitors, by adding 100 µL of medium containing inhibitors at the specified concentrations or DMSO alone at the specified dilutions. After 46-50 hours for VeroE6 and A549-hACE2 cells and after 70-74 hours for VeroE6 and Huh7.5 cells, cell viability was evaluated following the manufacturer’s guidelines. VeroE6 cells were tested with different incubation times to control for assays with 48 versus 72 hours incubation, respectively. 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% CO₂. 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 2 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 2 to 4 replicates with SEM. Sigmoidal concentration-response curves were fitted and 50% cytotoxic concentration (CC50) values were calculated using GraphPad Prism 8.0.0 with a bottom constraint of 0 applying the formula $Y = \frac{\text{Top}}{1+10^{(\log_{10}\text{EC50}-X)\cdot\text{HillSlope}}}$.  

Top/(1+10^{(Log_{10}\text{EC50}-X)\cdot\text{HillSlope}}).
Viral cytopathogenic effect (CPE) assay for determination of MOI for short-term treatment assays. To select a suitable MOI for short-term treatment assays not resulting in virus induced CPE (immunostaining-based assay) or relatively strong CPE (CPE-based assay) during the assays, viral CPE assays were carried out. Cells were plated on 96-well flat-bottom plates as described for short-term treatment assays. The following day cells were infected at different MOI with SARS-CoV-2/human/Denmark/DK-AHH1/2020 using 100 µL per well with 4 replicates per MOI. Following incubation times used in short-term treatment assays, CPE was evaluated using the Viral ToxGlo Assay (Promega) according to the manufacturer’s guidelines. Relative light units (RLU) from infected cultures were related to the mean RLU of noninfected control cultures to detect CPE.

Longer-term antiviral treatment assays in SARS-CoV-2 infected VeroE6 and A549-hACE2 cells. Cells were seeded at 10^6 cells per flask in T25 flasks (Nunc) and the following day infected at MOI 0.00002 (VeroE6 cells) or MOI 0.0005 (A549-hACE2 cells) with SARS-CoV-2/human/Denmark/DK-AHH1/2020. Cells were treated with specified fold EC50 of inhibitors on the day of infection, by adding inhibitors together with the virus and again on day 1 post infection. Following, cells were split and treated every 2 days with the specified concentrations of inhibitors and the percentage of infected culture cells was evaluated by immunostaining for the SARS-CoV-2 Spike protein and immunofluorescence imaging, as described below. In experiments with A549-hACE2 cells the concentration of DMSO was kept constant in all cultures. Upon cell splitting culture supernatants were harvested and stored at -80°C. Selected culture supernatants were used to determine viral RNA titers by RT-qPCR and to evaluate the presence of infectious virus using an infectivity assay. For each experiment a nontreated infected culture was included serving as a positive control for infection. Cultures were closed when massive cell death occurred, induced by viral infection and/or inhibitor treatment. Cell death was monitored in the light microscope.
Immunostaining and immunofluorescence imaging for evaluation of longer-term VeroE6 and A549-hACE2 cell cultures. In longer-term SARS-CoV-2 infected and PI treated cultures, following cell splitting and treatment, replicate cell cultures were seeded in 8-well chamber slides (Thermo Fisher Scientific, Rochester, NY, USA). The next day, cells were fixed, and virus was inactivated by submersion into methanol for 20 minutes. Chamber slides were washed twice with PBS-tween and then incubated with primary antibody SARS-CoV-2 spike chimeric monoclonal antibody (Sino Biological #40150-D004, Beijing, China) diluted 1:1,000 in PBSK for 2 hours at room temperature. Following 2 washes with PBS-tween, chamber slides were incubated 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 in PBS-tween for 20 minutes at room temperature. The percentage of SARS-CoV-2 Spike protein positive cells was evaluated by fluorescence microscopy (ZEISS Axio Vert.A1, Jena, Germany), assigning the following designations: 0% infected cells (no cells infected), single infected cells, and 10%–90% infected cells (in steps of 10%). The images were acquired with ZEN 3.0 software.

RT-qPCR assay for determination of SARS-CoV-2 RNA titers for evaluation of longer-term VeroE6 and A549-hACE2 cell cultures. For longer-term cultures, upon cell splitting and treatment, supernatant was harvested and stored at -80°C. Supernatant was mixed 1:3 with Trizol LS (Life Technologies) and RNA was extracted with chloroform (Sigma) using 5PRIME Phase Gel Lock Heavy tubes (Quantbio). RNA was purified using RNA Clean and Concentrator-5 kit (ZYMO Research) following manufacturer’s guidelines and RNA was eluted in nuclease-free water (Ambion). qPCR reactions were carried out using the TaqMan Fast Virus 1-Step Master Mix (Thermo Fischer) with previously described primers and probes:91 E_Sarbeco_F (5’-ACAGGTACGTTAATAGTTAATAGCGT-3’), E_Sarbeco_R (5’-ATATTGCAGCAGCAGCACA-3’) and E_Sarbeco_P (FAM-5’-).
ACACTAGCCATCCTTACTGCGCTTCG-3’-BHQ1). Primers were used at 400 nM and probe was used at 200 nM together with 2.5 µL purified RNA. Cycling conditions were as follows: For reverse transcription 10 minutes at 55°C, followed by 3 minutes at 95°C and 45 cycles of 95°C for 15 seconds and 58°C for 30 seconds using the LifeCycler 96 System (Roche). For every assay a negative control and RNA standards ranging from 10 to 10⁵ RNA copies per µL (Twist Bioscience) were included. RNA titers (genome copies/mL) were calculated by interpolation of cycle threshold values of the standard curve generated using the standard panel and the LightCycler software. The LLOQ of the assay was calculated as [(mean of RNA titers in supernatants derived from noninfected control cultures) + (3 standard deviations)].

**Infectivity assay for evaluation of presence of infectious virus in supernatants from longer-term VeroE6 cell cultures.** VeroE6 indicator cell cultures were seeded at 10,000 cells per well in 96-well flat-bottom plates. The following day the medium was exchanged with 100 µL of cell culture supernatants diluted 1:5 in cell culture medium. Culture supernatants were harvested every 2-3 days from replicate cultures not receiving treatment derived from longer-term VeroE6 cell cultures. Longer-term VeroE6 cell cultures treated with 3- or 5-fold EC50 boceprevir (Figure 8) or 1-fold EC50 simeprevir in combination with 0.4-fold EC50 remdesivir (Figure 9) were investigated. For each supernatant, 4 replicate indicator cultures were inoculated. 12 cultures inoculated with SARS-CoV-2/human/Denmark/DK-AHH1/2020 at MOI 0.01 served as a positive control for infection and 12 noninfected cultures served as negative controls. After 46-50 hours of incubation at 37°C and 5% CO₂, cells were subjected to immunostaining for the SARS-CoV-2 Spike protein and the number of single infected cells was evaluated by automated counting as described above.
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Author Contributions S.R., J.B. and J.M.G. conceived this project. K.A.G., Y.Z., A.G., S.R. and J.M.G. designed the experiments. K.A.G., Y.Z., C.R.D.H., A.G. and A.O. carried out the experiments. K.A.G., Y.Z. C.R.D.H. A.G., A.O., R.C. and J.M.G. analyzed and interpreted the data. K.A.G., Y.Z., A.O., R.C., L.P., U.F., S.F. T.K.H.S. and S.R. contributed to isolation and characterization of SARS-CoV-2/human/Denmark/DK-AHH1/2020 in vitro and established experimental systems. K.A.G., Y.Z. and J.M.G. prepared an initial manuscript draft. All authors contributed to and discussed the manuscript. J.M.G. supervised the study.

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**Figure legends**

**Figure 1.** Potency of a panel of HCV PI and an HCV NS4A inhibitor against SARS-CoV-2 in *VeroE6* cells. *VeroE6* cells were seeded in 96-well plates and the following day infected with SARS-CoV-2 at MOI 0.002 followed by treatment with specified concentrations of the PI boceprevir, telaprevir, narlaprevir, simeprevir, paritaprevir, grazoprevir, glecaprevir, voxilaprevir, vaniprevir, danoprevir, deldeprevir, asunaprevir and faldaprevir, as well as HCV NS4A inhibitor ACH-806, as described in Materials and Methods. After 46-50 hours of incubation, SARS-CoV-2 infected cells were visualized by immunostaining for the SARS-CoV-2 Spike protein and quantified by automated counting, as described in Materials and Methods. Datapoints (red dots) are means of counts from 7 replicate cultures ± standard errors of the means (SEM) and represent % residual infectivity, determined as % SARS-CoV-2 positive cells relative to means of counts from 14 replicate infected nontreated control cultures. Sigmoidal concentration-response curves (red lines) were fitted and EC50 values were determined, as described in Materials and Methods. Cell viability data were obtained in replicate assays with noninfected cells using a colorimetric assay, as described in Materials and Methods. Datapoints (blue triangles) are means of 3 replicate cultures ± SEM and represent % cell viability relative to mean absorbance from 12 replicate nontreated control cultures. Sigmoidal concentration-response curves were fitted and CC50 values were determined as shown in Supplemental Figure 4. The red dotted lines represent the drug concentrations at which DMSO is expected to induce antiviral effects with reduction of residual infectivity to <70%, according to Supplemental Figure 2. The blue dotted lines represent the drug concentrations at which DMSO is expected to induce cytotoxicity with reduction of cell viability to <90%, according to Supplemental Figure 2.

**Figure 2.** Potency of selected HCV PI against SARS-CoV-2 was confirmed in Huh7.5 cells. Huh7.5 cells were seeded in 96-well plates and the following day infected with SARS-CoV-2 at...
MOI 0.02 followed by treatment with specified concentrations of the PI boceprevir, simeprevir and grazoprevir, as described in Materials and Methods. After 70-74 hours incubation, SARS-CoV-2 infected cells were visualized by immunostaining for the SARS-CoV-2 Spike protein and quantified by automated counting, as described in Materials and Methods. Datapoints (red dots) are means of 7 replicates ± SEM and represent % residual infectivity, determined as % SARS-CoV-2 positive cells relative to means of counts from 14 replicate infected nontreated control cultures. Sigmoidal concentration-response curves (red lines) were fitted and EC50 values were determined, as described in Materials and Methods. Cell viability data were obtained in replicate assays with noninfected cells using a colorimetric assay as described in Materials and Methods. Data points (blue triangles) are means of 3 replicate cultures ± SEM and represent % cell viability relative to mean absorbance of 12 nontreated controls. Sigmoidal concentration-response curves were fitted and CC50 values were determined, as shown in Supplemental Figure 5. The blue dotted line represents the drug concentrations at which DMSO is expected to induce cytotoxicity with reduction of cell viability to <90%, according to Supplemental Figure 2; DMSO did not induce antiviral effects in the tested concentration ranges (Supplemental Figure 2).

**Figure 3. Potency of selected HCV PI against SARS-CoV-2 was confirmed in A549-hACE2 cells.** A549-hACE2 cells were seeded in 96-well plates and the following day infected with SARS-CoV-2 at MOI 0.003 followed by treatment with specified concentrations of the PI boceprevir, simeprevir, grazoprevir, glecaprevir and voxilaprevir, as described in Materials and Methods. After 46-50 hours incubation, SARS-CoV-2 infected cells were visualized by immunostaining for the SARS-CoV-2 Spike protein and quantified by automated counting, as described in Materials and Methods. Datapoints (red dots) are means of 7 replicates ± SEM and represent % residual infectivity, determined as % SARS-CoV-2 positive cells relative to means of counts from 14 replicate infected nontreated control cultures. Sigmoidal concentration-response curves (red lines)
were fitted and EC50 values were determined, as described in Materials and Methods. Cell viability data were obtained in replicate assays with noninfected cells using a colorimetric assay as described in Materials and Methods. Data points (blue triangles) are means of 3 replicate cultures ± SEM and represent % cell viability relative to mean absorbance of 12 nontreated controls. Sigmoidal concentration-response curves were fitted and CC50 values were determined, as shown in Supplemental Figure 6. The red dotted lines represent the drug concentrations at which DMSO is expected to induce antiviral effects with reduction of residual infectivity to <70%, according to Supplemental Figure 2. The blue dotted lines represent the drug concentrations at which DMSO is expected to induce cytotoxicity with reduction of cell viability to <90%, according to Supplemental Figure 2.

Figure 4. Analysis of interactions of selected HCV PI with remdesivir in VeroE6 cells. VeroE6 cells seeded in 96-well plates were infected the following day with SARS-CoV-2 at MOI 0.002 followed by treatment with serial dilutions of the linear PI boceprevir (BOC) or narlaprevir (NAR), or the macrocyclic PI simeprevir (SIM), paritaprevir (PAR) or grazoprevir (GRA), or polymerase inhibitor remdesivir (REM), or a combination of these PI and remdesivir, as described in Materials and Methods. After 46-50 hours incubation, SARS-CoV-2 infected cells were visualized by immunostaining for the SARS-CoV-2 Spike protein and quantified by automated counting, as described in Materials and Methods. For each inhibitor pair to be evaluated 7-10 treatment conditions were used (indicated on x-axis). Each treatment condition was defined by a given concentration of PI applied singly, a given concentration of remdesivir applied singly, and a combination of these same concentrations of PI and remdesivir, as specified in Supplemental Table 2, resulting in 3 datapoints per treatment condition. Datapoints are means of 6 or 7 replicates ± SEM and represent % residual infectivity, determined as % SARS-CoV-2 positive cells relative to means of counts from infected nontreated control cultures. Sigmoidal concentration-response curves...
Figure 5. Analysis of interactions of selected HCV PI with remdesivir in A549-hACE2 cells.

A549-hACE2 cells seeded in 96-well plates were infected the following day with SARS-CoV-2 at MOI 0.003 followed by treatment with serial dilutions of the linear PI boceprevir (BOC) or the macrocyclic PI simeprevir (SIM) or grazoprevir (GRA), or polymerase inhibitor remdesivir (REM), or a combination of these PI and remdesivir, as described in Materials and Methods. After 46-50 hours incubation, SARS-CoV-2 infected cells were visualized by immunostaining for the SARS-CoV-2 Spike protein and quantified by automated counting, as described in Materials and Methods. For each inhibitor pair to be evaluated 8-10 treatment conditions were used (indicated on x-axis).

Each treatment condition was defined by a given concentration of PI applied singly, a given concentration of remdesivir applied singly, and a combination of these same concentrations of PI and remdesivir, as specified in Supplemental Table 2, resulting in 3 datapoints per treatment condition. Datapoints are means of 7 replicates ± SEM and represent % residual infectivity, determined as % SARS-CoV-2 positive cells relative to means of counts from infected nontreated control cultures. Sigmoidal concentration-response curves were fitted as described in Materials and Methods. DMSO was kept constant in all cultures. The tested inhibitor concentrations did not impair cell viability (Supplemental Figure 8). DMSO did not induce antiviral effects in the tested concentration ranges (Supplemental Figure 2).

Figure 6. Comparison of barrier to escape for HCV PI at equipotent concentrations in VeroE6 cells. VeroE6 cells seeded the previous day in T25 flasks were infected with SARS-CoV-2 at MOI 0.00002 followed by treatment with 1-fold EC50 of boceprevir, telaprevir, narlaprevir, simeprevir, paritaprevir, grazoprevir, vaniprevir, danoprevir, asunaprevir and faldaprevir, which...
were administered immediately after infection and subsequently at the indicated timepoints post infection when cells were split, as described in Materials and Methods. Left panel, the % of SARS-CoV-2 infected cells on the specified days post infection was determined by anti-Spike protein immunostaining of replicate cultures derived following cell splitting and treatment. Middle panel, SARS-CoV-2 RNA titers determined in cell culture supernatants as genome copies/mL on the specified days post infection were determined by RT-qPCR assays. The black line indicates the LLOQ. In the left and middle panel, to facilitate comparisons, bars are color coded according to the day post infection and blue and red dotted lines were inserted to highlight day 1 and 3 values of the nontreated culture, respectively. Right panel, replicate cultures were derived following cell splitting and treatment and immunostained for the SARS-CoV-2 Spike protein (green) and counterstained with Hoechst dye (blue), and images were acquired, as described in Materials and Methods. Cultures summarized in this figure are derived from different experimental setups, each including an infected nontreated control culture, which showed viral spread comparable to that in the depicted representative culture. *Culture was terminated, or infection data not recorded, due to virus induced cell death. **Culture was terminated due to drug induced cytotoxicity, possibly enhanced by viral infection.

**Figure 7. Comparison of barrier to escape for HCV PI in A549-hACE2 cells.** A549-hACE2 cells seeded the previous day in T25 flasks were infected with SARS-CoV-2 at MOI 0.0005, followed by treatment with indicated concentrations of specified inhibitors administered immediately after infection and subsequently at the listed timepoints when cells were split, as described in Materials and Methods. BOC, boceprevir; SIM, simeprevir; GRA, grazoprevir; REM, remdesivir. Upon splitting of cells, cell culture supernatant was harvested and subjected to RT-qPCR for determination of SARS-CoV-2 RNA titers determined as genome copies/mL. The black line indicates the LLOQ. In order to facilitate comparisons, bars are color coded according to the
day post infection and blue and red dotted lines were inserted to highlight day 1 and 3 values of the nontreated culture, respectively. Cultures summarized in this figure are derived from different experimental setups, each including an infected nontreated control culture, which showed viral spread comparable to that in the depicted representative culture. Left panel, treatment with 1-fold EC50 boceprevir, simeprevir or grazoprevir. Middle panel, treatment with 1-, 2-, 3-, 4-, 5- and 8-fold EC50 boceprevir. * Culture was terminated due to virus or drug induced cytotoxicity. * Culture was maintained for a total of 13 days without indication of infection (RNA titers were around the LLOQ and no observation of single SARS-CoV-2 Spike protein positive cells). Right panel, treatment with 0.8-fold EC50 remdesivir, 1-fold EC50 boceprevir, 1-fold EC50 simeprevir, or 1-fold EC50 grazoprevir singly, or with a combination of remdesivir with either PI.

**Figure 8. Boceprevir was capable of completely suppressing SARS-CoV-2 in VeroE6 cells.**

VeroE6 cells seeded the previous day in T25 flasks were infected with SARS-CoV-2 at MOI 0.00002 followed by treatment with 1-, 1.5-, 2-, 2.5-, 3- and 5-fold EC50 boceprevir, which was administered immediately after infection and subsequently at the indicated timepoints when cells were split, as described in Materials and Methods. Left panel, the % of SARS-CoV-2 infected cells on the specified days post infection was determined by anti-Spike protein immunostaining of replicate cultures derived following cell splitting and treatment. Middle panel, SARS-CoV-2 RNA titers determined in cell culture supernatants as genome copies/mL on the specified days post infection were determined by RT-qPCR assays. The black line indicates the LLOQ. In the left and middle panel, to facilitate comparisons, bars are color coded according to the day post infection and blue and red dotted lines were inserted to highlight day 1 and 3 values of the nontreated culture, respectively. Right panel, replicate cultures were derived following cell splitting and treatment, and immunostained for the SARS-CoV-2 Spike protein (green) and counterstained with Hoechst dye (blue), and images were acquired, as described in Materials and Methods. Cultures summarized in
this figure are derived from different experimental setups, each including an infected nontreated control culture, which showed viral spread comparable to that in the depicted representative culture. *Culture was terminated, or infection data not recorded, due to virus induced cell death. †. Culture was maintained for a total of 17 days without indication of infection (no observation of single SARS-CoV-2 Spike protein positive cells and RNA titers were around the LLOQ).

**Figure 9. At equipotent concentrations, simeprevir and grazoprevir but not boceprevir synergized with remdesivir to completely suppress viral infection in VeroE6 cells.** VeroE6 cells seeded the previous day in T25 flasks were infected with SARS-CoV-2 at MOI 0.00002 followed by treatment with 0.4-fold EC50 remdesivir (REM), 1-fold EC50 boceprevir (BOC), 1-fold EC50 simeprevir (SIM) or 1-fold EC50 grazoprevir (GRA) singly, or with a combination of remdesivir with either PI, which was administered immediately after infection and subsequently at the indicated timepoints when cells were split, as described in Materials and Methods. Left panel, the % of SARS-CoV-2 infected cells on the specified days post infection was determined by anti-Spike protein immunostaining of replicate cultures derived following cell splitting and treatment. Middle panel, SARS-CoV-2 RNA titers determined in cell culture supernatants as genome copies/mL on the specified days post infection were determined by RT-qPCR assays. The black line indicates the LLOQ. In the left and middle panel, to facilitate comparisons, bars are color coded according to the day post infection and blue and red dotted lines were inserted to highlight day 1 and 3 values of the nontreated culture, respectively. Right panel, replicate cultures were derived following cell splitting and treatment, and immunostained for the SARS-CoV-2 Spike protein (green) and counterstained with Hoechst dye (blue), and images were acquired, as described in Materials and Methods.

Cultures summarized in this figure are derived from two different experimental setups (REM/BOC/SIM and REM/GRA experiments), each including the respective depicted nontreated control culture. *Culture was terminated, or infection data not recorded, due to virus induced cell death.
death. Culture was maintained for a total of 15 days without indication of infection (no observation of single SARS-CoV-2 Spike protein positive cells and RNA titers were around the LLOQ).
Table 1. Potency of a panel of HCV PI and an HCV NS4A inhibitor against SARS-CoV-2 in vitro

| VeroE6 cells | EC50 (µM)a | CC50 (µM)b | SIc |
|--------------|------------|------------|-----|
| Inhibitor    |            |            |     |
| Boceprevir   | 44         | >1214      | >28 |
| Telaprevir   | 40         | >432       | >11 |
| Narlaprevir  | 37         | 269        | 7.3 |
| Simeprevir   | 15         | 59         | 3.9 |
| Paritaprevir | 22         | 123        | 5.6 |
| Grazoprevir  | 42         | 239        | 5.7 |
| Glecaprevir  | >178       | >268       | n.d.|
| Voxilaprevir | >27        | 72         | <2.6|
| Vaniprevir   | 51         | 171        | 3.4 |
| Danoprevir   | 87         | >243       | >2.8|
| Deldeprevir  | >20        | 56         | <2.8|
| Asunaprevir  | 72         | 263        | 3.7 |
| Faldaprevir  | 23         | 246        | 6.3 |
| ACH-806      | 46         | >429       | >9.3|

| Huh7.5 cells | EC50 (µM)a | CC50 (µM)b | SIc |
|--------------|------------|------------|-----|
| Inhibitor    |            |            |     |
| Boceprevir   | 42         | 701        | 33  |
| Simeprevir   | 14         | 33         | 2.4 |
| Grazoprevir  | 20         | 133        | 6.7 |

| A549-hACE2 cells | EC50 (µM)a | CC50 (µM)b | SIc |
|------------------|------------|------------|-----|
| Inhibitor        |            |            |     |
| Boceprevir       | 20         | >1213      | >61 |
| Simeprevir       | 9          | 56         | 6.2 |
| Grazoprevir      | 26         | 125        | 4.8 |
| Glecaprevir      | >94        | >268       | n.d.|
| Voxilaprevir     | 10         | 81         | 8.1 |

EC50, 50% effective concentration (µM), determined in antiviral treatment assays as described in Materials and Methods. For voxilaprevir and deldeprevir, in VeroE6 cells, >50% residual infectivity was observed at the highest noncytotoxic concentrations; for glecaprevir in both VeroE6 cells and A549-hACE2 cells, the highest applied concentration was limited due to antiviral effects of the
diluent DMSO; thus, for these PI no precise EC50 value could be determined. EC50 values are also included in Figures 1, 2 and 3.

`CC50, 50% cytotoxic concentration (µM), determined in cell viability assays as described in Materials and Methods. For boceprevir, telaprevir, glecaprevir, danoprevir and ACH-806, in VeroE6 cells and for boceprevir and glecaprevir in A549-hACE2 cells, >50% cell viability was observed at the highest concentrations tested; thus, no precise CC50 could be determined. Tested were concentrations at which DMSO was not expected to reduce cell viability to <90% (Supplemental Figure 2).

SI, selectivity index, determined as CC50/EC50 based on results from antiviral treatment assays and cell viability assays. n.d., not determined.
Figure 2

**Boceprevir**
- EC50 = 42
- CC50 = 701

**Simeprevir**
- EC50 = 14
- CC50 = 33

**Grazoprevir**
- EC50 = 20
- CC50 = 133

![Graphs showing the inhibitory effect of Boceprevir, Simeprevir, and Grazoprevir on SARS-CoV-2 and cell viability.](https://journals.asm.org/journal/aac)
Figure 3

For Boceprevir:
- EC50 = 20
- CC50 = >1213

For Simeprevir:
- EC50 = 9
- CC50 = 56

For Grazoprevir:
- EC50 = 26
- CC50 = 125

For Glecaprevir:
- EC50 = >94
- CC50 = 268

For Voxilaprevir:
- EC50 = 10
- CC50 = 81
Figure 4

The figure shows a series of graphs representing the residual infectivity of SARS-CoV-2 under different treatment conditions. Each graph plots residual infectivity (%) against treatment condition.

- **BOC + REM**
  - Treatment Condition: From 1 to 8
  - Indicates a significant reduction in residual infectivity.

- **NAR + REM**
  - Treatment Condition: From 1 to 10
  - Shows a gradual decrease in residual infectivity.

- **SIM + REM**
  - Treatment Condition: From 1 to 8
  - Displays a sharp decrease in residual infectivity.

- **PAR + REM**
  - Treatment Condition: From 1 to 7
  - Exhibits a moderate decrease in residual infectivity.

- **GRA + REM**
  - Treatment Condition: From 1 to 10
  - Demonstrates a consistent decrease in residual infectivity.
Figure 5

Graphs showing the residual infectivity of SARS-CoV-2 under different treatment conditions.

Top graph:
- BOC
- REM
- BOC+REM

Middle graph:
- SIM
- REM
- SIM+REM

Bottom graph:
- GRA
- REM
- GRA+REM

Treatment Conditions range from 1 to 10.
Figure 7
Figure 8

[Graph showing data on percentage SARS-CoV-2 Spike protein positive cells and SARS-CoV-2 genome copies/mL for different treatments over days 1 to 9.]
