Potential anti-COVID-19 agents, cepharanthine and nelfinavir, and their usage for combination treatment
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SUMMARY
Antiviral treatments targeting the coronavirus disease 2019 are urgently required. We screened a panel of already approved drugs in a cell culture model of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and identified two new agents having higher antiviral potentials than the drug candidates such as remdesivir and chloroquine in VeroE6/TMPRSS2 cells: the anti-inflammatory drug cepharanthine and human immunodeficiency virus protease inhibitor nelfinavir. Cepharanthine inhibited SARS-CoV-2 entry through the blocking of viral binding to target cells, while nelfinavir suppressed viral replication partly by protease inhibition. Consistent with their different modes of action, synergistic effect of this combined treatment to limit SARS-CoV-2 proliferation was highlighted. Mathematical modeling in vitro antiviral activity coupled with the calculated total drug concentrations in the lung predicts that nelfinavir will shorten the period until viral clearance by 4.9 days and the combining cepharanthine/nelfinavir enhanced their predicted efficacy. These results warrant further evaluation of the potential anti-SARS-CoV-2 activity of cepharanthine and nelfinavir.

INTRODUCTION
The novel coronavirus disease 2019 (COVID-19), caused by the infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a global public health problem that is impacting social and economic damage worldwide (Huang et al., 2020; Zhou et al., 2020; Zhu et al., 2020). More than 5,000,000 confirmed cases with over 300,000 deaths were reported late May 2020 across 216 countries/areas/territories (WHO, 2020). COVID-19 was characterized as a pandemic by the World Health Organization (WHO), and new treatments along with a vaccine are urgently needed. Remdesivir (RDV), a nucleoside analog originally developed for treating Ebola virus with several other Food and Drug Administration (FDA)-approved drugs, is being evaluated in patients with COVID-19, including lopinavir (LPV) boosted by ritonavir, chloroquine (CQ), favipiravir (FPV), and interferon (Beigel et al., 2020; Boulware et al., 2020; Cao et al., 2020; Dong et al., 2020; Touret and de Lamballerie, 2020). Reports on the clinical efficacies of these drugs are pending; however, it would be prudent to have a pipeline of additional drug candidates available for clinical trials.

In this study, we screened a panel of already approved drugs in a SARS-CoV-2 infection cell culture assay and identified two, cepharanthine (CEP) and nelfinavir (NFV), that showed more potent antiviral activity compared to RDV and other drugs currently being trialed. Our in vitro, in silico, and cell culture analyses demonstrate that CEP and NFV inhibit SARS-CoV-2 entry and RNA replication, respectively. Their different modes of action provided synergistic antiviral effects. We also mathematically predicted the potential antiviral efficacy of the single treatment of either CEP or NFV and its combination in clinical settings. These data cumulatively provide evidence for anti-SARS-CoV-2 potentials of CEP and NFV.

Continued
RESULTS

Cepharanthine and nelfinavir inhibit SARS-CoV-2 infection

We established a cell-based drug screening system to identify compounds that protect cells from SARS-CoV-2-induced cytopathology (Figure 1A): VeroE6/TMPRSS2 cells were treated with compounds for 1 hr during inoculation with a clinical isolate of SARS-CoV-2 (Matsuyama et al., 2020) at a multiplicity of infection (MOI) of 0.01 (or 0.001 for the indicated assay). Unbound virus was removed by washing, and the cells were treated with compounds for 48 hr to assess cell viability (Figure 1A) (methods). SARS-CoV-2 replication in VeroE6/TMPRSS2 induced a cytopathic effect and to validate our assay we show that two compounds, LPV and CLQ, that were reported to inhibit SARS-CoV-2 infection (Choy et al., 2020; Pizzorno et al., 2020; Wang et al., 2020a) reduced virus-induced cytopathicity (Figure 1B, compare b and c, d). After screening 306 FDA/European Medicines Agency/Pharmaceuticals and Medical Devices Agency-approved drugs, we identified compounds that protected cell viability by 20-fold compared with a dimethyl sulfoxide solvent control (methods). Among these, we selected to study CEP and NFV as candidates showing the greatest anti-cytotoxic activity (Figures 1B–g, h). CEP is a Stephania-derived alkaloid extract with anti-inflammatory and anti-oxidative activities, and NFV targets human immunodeficiency virus protease (Bailly, 2019; Kao et al., 2013; Markowitz et al., 1998). To confirm and extend these observations, we assessed SARS-CoV-2-encoded N protein expression 24 hr after inoculation by immunofluorescence (Figure 1C, red) and immunoblotting (Figure 1D). Both CEP and NFV significantly reduced N protein expression along with the positive control drug candidates LPV, CLQ, and RDV. We confirmed that CEP and NFV inhibit SARS-CoV-2 proliferation in a human-derived lung epithelial cell line Calu-3 cells (Figure 1E).

Dose-response curve for the anti-SARS-CoV-2 activity of cepharanthine and nelfinavir

To extend these observations, we quantified the effect of these compounds on secreted viral RNA and cell viability at 24 hr after infection. CEP and NFV significantly reduced viral RNA levels in a dose-dependent manner to 0.001–0.01% of the untreated control infections (Figure 2A). As expected, the positive control compounds (CLQ, LPV, and RDV) inhibited viral RNA, whereas FPV up to 64 \( \mu M \) showed negligible antiviral activity, consistent with previous reports (Choy et al., 2020; Jeon et al., 2020; Wang et al., 2020a). In parallel, we also assessed cell viability and noted cell death at high drug concentrations up to 64 \( \mu M \) (Figure 2B). The concentrations of drugs required to inhibit 50% (IC\(_{50}\)) or 90% (IC\(_{90}\)) of virus proliferation along with their 50% cytotoxicity (CC\(_{50}\)) were estimated by median effect model and are listed in Figures 2A and 2B. These experiments highlight a >70-fold win-}

Cepharanthine and nelfinavir have different modes of action

To determine how these compounds impact on the viral replicative life cycle, we performed a time-of-addition assay (Figure 3A). We measured the antiviral activity of drugs added at different times: (a) present during the 1 hr virus inoculation step and maintained throughout the 24 hr infection period ("whole life cycle"); (b) present during the 1 hr virus inoculation step and for an additional 2 hr and then removed ("entry"); or (c) added after the inoculation step and present for the remaining 22 hr of infection ("post-entry"). CLQ, a known modulator of intracellular pH that inhibits virus entry (Akpovwa, 2016), was recently reported to inhibit SARS-CoV-2 (Liu et al., 2020a; Wang et al., 2020a), and we confirmed its activity in the early stages of infection (Figure 3B, lane 5). Since this assay allows multiple rounds of re-infection, entry inhibitors can show antiviral effects when added post-entry as in protocol (c) (Figure 3B, lane 6). RDV was previously reported to inhibit the process for intracellular viral replication (Wang et al., 2020a), and we confirmed this mode of action showing a reduction in viral RNA levels with a negligible effect on virus entry (Figure 3B, lane 8). This assay identified that CEP targeted the virus entry phase (Figure 3B, lanes 11) while NFV clearly inhibited the post-entry process (Figure 3B, lanes 15).

Cepharanthine inhibits SARS-CoV-2 binding

In silico docking simulation shows that CEP molecules can bind the SARS-CoV-2 spike (S) protein and interfere with S engagement to its receptor, angiotensin-converting enzyme 2 (ACE2) (La et al., 2020; Walls et al., 2020; Wang et al., 2020b) (Figure 4A, green stick). The docking model suggests that the NH of the piperidine ring of CEP molecules forms a hydrogen bond with the side chain carboxyl group of Glu484 and the backbone carboxyl group of Ser494, and the aromatic rings are in close contact with the aromatic residues (Tyr449, Tyr453, Tyr489, and Phe490) at the binding interface with ACE2. Binding free energy of CEP molecules was estimated as −24.26 kcal/mol using
molecular mechanics generalized Born surface area calculation (Schrödinger, LLC). To assess this model, we investigated whether CEP inhibits SARS-CoV-2 particle binding to the cell surface or subsequent internalization into cells. We measured viral binding to cells by pre-chilling cells to prevent particle endocytosis and quantified cell-bound virus particles by quantitative PCR (qPCR) of viral RNA. CEP significantly inhibited SARS-CoV-2 binding to cells, whereas CLQ that targets intracellular trafficking pathways (Liu et al., 2020a) had a negligible effect (Figures 4B and S1). Viruses frequently exploit cellular heparan sulfate proteoglycans to initiate cell attachment, and heparin shows broad-spectrum inhibition of virus-cell attachment (De Clercq, 1998; Lang et al., 2011). As expected, heparin significantly blocked SARS-CoV-2 particle attachment in the infected cells at 24 hr after infection, and the red and blue signals show N and DAPI, respectively. These data were from three independent experiments.

**Nelfinavir potently targets SARS-CoV-2 main protease**

We conducted *in silico* docking simulation screenings to identify compounds from an approved library that interact with the SARS-CoV-2-encoded main protease (*methods*). Interestingly, NFV was identified among
the top 1.5% ranking compounds (Figure 5A, cyan stick: NFV, green: main protease). Our docking model predicts that NFV interacts with the SARS-CoV-2 protease active site pocket and would block substrate recruitment (Figure 5A). To assess this model, we evaluated the activity of recombinant SARS-CoV-2 main protease using an in vitro protease assay (methods). We showed that NFV inhibited the catalytic activity of the SARS-CoV-2-encoded main protease in a dose-dependent manner, and its IC50 was calculated to be 37 μM (Figure 5B). These in vitro and in silico data suggest that NFV potentially targets the main protease, but its inhibition activity is likely to be weaker than that to block SARS-CoV-2 replication.

Synergy between cepharanthine and nelfinavir in blocking SARS-CoV-2 infection

Both CEP and NFV show anti-SARS-CoV-2 activity at the concentration ranges observed in patients, where the serum Cmax of both drugs is 2.3 and 6.9 μM (by administration of 500 mg NFV orally and of 100 mg CEP by intravenous injection), respectively (Markowitz et al., 1998; Yasuda et al., 1989). Since CEP and NFV have different mode of actions, we examined their potential for synergistic effects. Antiviral activity and cell viability were determined by qPCR enumeration of viral RNA and MTT activity, respectively, following treatment with each compound alone or in combination (Figures 6A and 6B). For these experiments, we infected cells with lower amounts of SARS-CoV-2 (MOI = 0.001) and treated compounds at more frequent points of concentrations than those used in our earlier assay, for securing an accurate estimation. Single treatment with CEP (see white bars in Figure 6A) or NFV (see bars at CEP 0 μM) reduced viral RNA to 6.3% or 5.8% of untreated control, respectively, when combined they reduced viral RNA level to 0.068%. Higher doses of the CEP/NFV combination (4 μM each) reduced the viral RNA to undetectable levels. We compared the observed experimental antiviral activity (Figures 6A and S2A) with theoretical predictions calculated using a classical Bliss independence method.
that assumes drugs act independently (Note S1, Figure S2B) (Greco et al., 1995; Koizumi and Iwami, 2014). The difference between the observed values and theoretical predictions suggests that CEP and NFV exhibit a synergistic activity over a broad range of concentrations (Figure 6C red: synergistic effect).

**Mathematical modeling for the impact of cepharnanthine and nelfinavir on SARS-CoV-2 dynamics in clinical settings**

Combining the published human clinical pharmacokinetics information for these drugs (Markowitz et al., 1998; Yasuda et al., 1989; Yokoshima et al., 1986) with our observed dose-dependent antiviral data, we can calculate the antiviral activity at the time after administration (Figure 7A: left, NFV oral; center, CEP intravenous drip; right: CEP oral). Here, we used the reported pharmacokinetic information for drug distribution in the lung as well as the time-dependent drug concentration in plasma and assumed that antiviral activity depends on drug concentration in the lung (Ford et al., 2004; Shetty et al., 1996; Twigg et al., 2010) (see supplemental information in detail). Based on the time-dependent antiviral activity of drug, we can model the impact on viral burden following drug administration (Figure 7B, Note S1, Figure S3). From the viral dynamics data in Figure 7B, we calculated the cumulative viral RNA burden (i.e., area under the curve of viral load) (Figure 7C, upper) and the time required to reduce the viral load to undetectable levels (Figure 7C, lower). Our modeling predicts that NFV monotherapy would reduce the cumulative viral load by 92.1% (Figure 7C, upper, red) and would require 10.3 days to eliminate virus (Figure 7B, upper left, red), 4.9 days shorter than untreated controls (Figure 7C, lower, red). In contrast, orally administered CEP shows a minimal effect on the viral load (Figure 7B, lower left, green), most likely reflecting low drug concentration, while intravenous delivery of CEP reduces the cumulative viral load (Figures 7B and 7C, green) and shortens the period for virus elimination (Figure 7C, lower, green) because of achieving enough drug concentration (see Discussion). Importantly, co-administering NFV (oral) and CEP (intravenous drip) resulted in a more rapid decline in viral RNA, with undetectable levels 6.15 days earlier than untreated controls and 1.23 days earlier than NFV alone (Figure 7C, orange). Another advantage of combination treatment is discussed in discussion. In summary, our prediction shows the potential antiviral efficacy of NFV and CEP and its combined treatment that facilitates SARS-CoV-2 elimination.

**DISCUSSION**

Screening a panel of approved drugs identified two agents, CEP and NFV, that inhibit SARS-CoV-2 infection with the highest potencies in our screening. A recent study reported that CEP showed anti-SARS-CoV-2 activity, and the authors speculated that CEP targeted both entry and viral replication phase of the virus life cycle (Fan et al., 2020; Jeon et al., 2020). However, our time-of-addition studies along with viral binding and docking simulation analysis suggest that CEP predominantly inhibits virus-cell binding. We also have preliminary data by surface plasmon resonance analysis showing a potential interaction between CEP and the S protein, speculating its mode of action and which needs to be further analyzed in the future. These data are consistent with a previous paper reporting that CEP reduced the entry of another human
Coronavirus OC43 (Kim et al., 2019). There is a significant global effort to generate a COVID-19 vaccine that will target the SARS-CoV-2 S glycoprotein (Thanh Le et al., 2020). It is worthy of future investigation to examine whether CEP is effective to augment the antiviral activity of neutralizing antibodies. After the emergence of COVID-19 pandemic, in silico studies have been widely conducted to seek for anti-COVID-19 drugs and NFV was predicted for a potential to associate with SARS-CoV-2 life cycle (Huynh et al., 2020; Mittal et al., 2020; Mothay and Ramesh, 2020; Musarrat et al., 2020; Reiner et al., 2020). NFV was reported to inhibit the replication of another coronavirus, SARS-CoV (Liu et al., 2005; Wu et al., 2004; Yamamoto et al., 2004). Our study is consistent with the recent report showing the anti-SARS-CoV-2 activity of NFV, which has been published during the review process of this paper, although its mode of action and the prediction of antiviral effect in clinical settings were not analyzed (Ianevski et al., 2020).

In addition to the identification of anti-SARS-CoV-2 activity of NFV from a chemical screening, our study showed that NFV inhibited SARS-CoV-2 replication with under \( M \) order and inhibited the catalytic activity of main protease with lower activity, predicted by in silico modeling. Our data suggest that NFV potently inhibits the main protease and also possibly targets another factor. A non-infectious cell fusion system also reported that NFV inhibited SARS-CoV-2 spike-mediated membrane fusion at the concentration of over 10 \( \mu M \) (Musarrat et al., 2020), providing another possible antiviral activity of NFV. In addition, the observation that CEP and NFV target different steps in the viral life cycle supports the development of multidrug combination therapies for treating COVID-19.

Our mathematical modeling studies assess how drug candidates can suppress and eliminate SARS-CoV-2. Based on the reported lung distribution/concentration of CEP and NFV in patients, we predicted that NFV at clinical doses can maintain significant antiviral effects throughout the treatment period and reduce SARS-CoV-2 RNA burden that results in shortening the time required to eliminate infection. In contrast, we predict that oral administration of CEP will have limited antiviral effect due to its low concentration.

**Figure 4. CEP inhibits SARS-CoV-2 cell binding**

(A) Predicted binding of CEP molecule to SARS-CoV-2 spike protein. Spike protein, CEP molecule, and protein binding site residues around CEP within 4 \( \AA \) are shown in cartoon representation colored in orange, green stick, and surface representation, respectively. An overlapping view of the ACE2 with CEP is shown in semi-transparent cartoon representation colored in cyan.

(B) Virus-cell binding assay. VeroE6/TMPRSS2 cells were incubated with virus (MOI = 0.001) in the presence of the indicated compounds for 30 min at 4°C to allow virus-cell binding. After extensive washing, cell-bound viral RNA was quantified, where the background depicts residual viral inocula in the absence of cells (B; mean ± SD). These data were from three independent experiments. **p < 0.01; N.S., not significant (Student’s t-test)
in vivo. However, intravenous delivery of CEP achieves higher drug concentrations especially accumulated in the lung (Yokoshima et al., 1986) that enables sustained antiviral activity. It is noteworthy that combining CEP with NFV further reduced the cumulative viral load and facilitated virus elimination. As the cumulative viral load in patients is likely to associate with disease progression and risk of new transmission (Liu et al., 2020b), such multidrug treatments will be of benefit to improve clinical outcome and to control the epidemic. In addition to potentiating antiviral effects, combination treatment can limit the emergence of viral drug resistance which is frequently reported for RNA viruses such as coronavirus. Limitations of this mathematical prediction are shown in limitations of the study section; however, our analysis warrants the further clinical trial for oral NFV treatment in Japan (jRCT2071200023).

Several in vivo SARS-CoV-2 infection systems were recently reported: nonhuman primates, ferrets, hamsters, transgenic mice overexpressing human ACE2, and wild-type mice infected with mouse-adapted virus (Bao et al., 2020; Gao et al., 2020; Kim et al., 2020; Munster et al., 2020; Rockx et al., 2020). Given the urgency of the COVID-19 pandemic, we believe that a lack of in vivo validation should not preclude the clinical assessment of new antiviral agents. We here propose CEP and NFV as potential antiviral drug candidates against COVID-19, and thus, NFV is under clinical evaluation in a multicenter randomized controlled trial in Japan (jRCT2071200023).

Limitations of the study
In this study, we mainly used VeroE6/TMPRSS2 cells and applied the dose-dependent antiviral activity in these cells (Figure 2A) to predict the drug efficacy in patients (Figure 7). More physiologically relevant cell models such as primary human respiratory/lung cells in air-liquid interface culture and organoids or presumably in vivo infection models would be needed to strengthen the data. As well, our mathematical prediction was based on the total drug concentration in the lung, although free drug that does not non-specifically bind to proteins is believed to be pharmacologically active. There is no information available on the free CEP and NFV concentration in the lung tissue.

Resource availability
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Koichi Watashi: kwatashi@nih.go.jp.
Figure 6. Combination treatment with CEP and NFV

(A) Dose-response curve of CEP/NFV co-treatment in the infection experiment (MOI = 0.001). Extracellular viral RNA levels at 24 hr after infection were quantified and plotted against concentrations of CEP (0.78, 1.25, 2.00, 3.20, and 5.12 μM: 1.6-fold serial dilution) and NFV (1.08, 1.30, 1.56, 1.87, and 2.24 μM: 1.2-fold serial dilution).

(B) Cell viability upon co-treatment with compounds.

(C) The three-dimensional interaction landscapes of CEP and NFV were evaluated based on the Bliss independence model. Red and blue colors on the contour plot indicate synergy and antagonism, respectively. These data were from three independent experiments (A, B; mean ± SD).

Materials availability
This study did not generate new unique materials.

Data and code availability
All data are included in the article and supplemental information and any additional information will be available from the lead contact upon request.

METHODS
All methods can be found in the accompanying transparent methods supplemental file.
Figure 7. Mathematical prediction of the impact of CEP and NFV therapy on viral dynamics

(A) The time-dependent antiviral effects of NFV [500 mg, TID, oral] and CEP [25 mg, intravenous drip or 10 mg, oral] predicted by pharmacokinetics/pharmacodynamics (PK/PD) model are shown, with enlarged views of the gray zones in upper panels.

(B) Viral load dynamics in the presence or absence of NFV (oral), CEP (intravenous), CEP (oral), and NFV (oral)/CEP (intravenous) combined therapies predicted by pharmacokinetics/pharmacodynamics/viral dynamics (PK/PD/VD) models are shown.

(C) The cumulative antiviral load [area under the curve in (B)] (upper) and the reduction time (days) for virus elimination (lower) with drug treatments [NFV (oral), CEP (intravenous), and the NFV (oral)/CEP (intravenous) combination] are shown.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102367.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, K.W.; investigation, H.O., K.W., W.S., K.S., S. Iwami, T.H., T. Shirai, S.K., Y.I., K.S.K., T.N., Tateki Suzuki, K.N., and S. Iwami; methodology and resources, S.Ando., Tadaki Suzuki., T.H., K.M., M.S., M.T., T.W.; analysis, all the authors; writing and editing, K.W., T.H., K.A., S. Iwami, and J.A.M; funding acquisition, K.W. and M.T.; supervision, K.W.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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Supplemental information

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Potential anti-COVID-19 agents, Cepharanthine and Nelfinavir, and their usage for combination treatment

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Cell culture. VeroE6/TMPRSS2 cells (VeroE6 cells overexpressing transmembrane protease, serine 2 (TMPRSS2) (Matsuyama et al., 2020) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Cell Culture Bioscience), 100 units/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES (pH 7.4), and 1 mg/mL G418 (Nacalai) at 37˚C in 5% CO₂. During the infection assay, 10% FBS was replaced with 2% FBS and G418 removed. Calu-3 cells were cultured in above medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Reagents. All the reagents were purchased from Selleck, Enzo Life Sciences, Cayman Chemical, Sigma, MedChemExpress, TCI or kindly donated by pharmaceutical companies (Abbvie, Alps Pharmaceutical, Asahi Kasei Pharma, Astellas Pharma, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai Pharmaceutical, Daiichi Sankyo, EA Pharma, Fujifilm Toyama Chemical, Japan Tobacco, Kakenshoyaku, Kissei Pharmaceutical, Kowa, Kyorin Pharmaceutical, Kyowa Pharmaceutical Industry, Maruho, Mitsubishi Tanabe Pharma, Mochida Pharmaceutical, Novartis, Sanofi, SBI Pharmaceuticals, Shionogi, Sumitomo Dainippon Pharma, Sun Pharma, Takeda Pharmaceutical, Teva Takeda Pharma). Note that throughout in this study we used the pharmaceutical preparation of Cepharanthine (kindly provided by Medisa Shinyaku Inc, a subsidiary of Sawai Pharmaceutical), which is a Stephania-derived alkaloid extract containing 19.5-33.5% Cepharanthine molecule as the major component.

Infection assay. SARS-CoV-2 was handled in a biosafety level 3 (BSL3). We used the SARS-CoV-2 Wk-521 strain, a clinical strain isolated from a COVID-19 patient, and obtained viral stocks by infecting VeroE6/TMPRSS2 cells (Matsuyama et al., 2020). Virus infectious titers were measured by inoculating cells with a 10-fold serial dilution of virus and cytopathology measured to calculate TCID₅₀/ml (Matsuyama et al., 2020). For the infection assay, VeroE6/TMPRSS2 cells were inoculated with virus at an MOI of 0.01 (Fig. 1, 2, and 3B, except for 0.001 in Fig. 1B) for 1 h and unbound virus removed by washing. In Fig. 4B, 6, and S1, we used 0.001 of MOI to avoid possible saturation of virus binding/replication. Cells were cultured for 24 h prior to measuring extracellular viral RNA or detecting viral encoded N protein, and cytopathic effects (CPE) after 48 h. Compounds were added during virus inoculation (1 h) and replenished after washing (24 or 48 h) except for time of addition assay. Infection assay with Calu-3 cells was performed by incubation with virus at an MOI of 0.04 for 3 h. N protein was detected at 72 h post-inoculation.

For the time of addition assay, we added compounds with three different timings (Fig. 3A): (a) present during the 1 h virus inoculation step and maintained throughout the 24 h infection period (whole life cycle); (b) present during the 1 h virus inoculation step and for an additional 2 h and then removed (entry); or (c) added after the inoculation step and present for the remaining 22 h of infection (post-entry). Inhibitors of viral replication are expected to show antiviral activity in (a) and (c), but not (b), while entry inhibitors (e.g. chloroquine) reduce viral RNA in all three conditions (In c, addition of entry...
inhibitors after inoculation inhibits re-infection and thus decreases viral RNA) (Wang et al., 2020).

**Quantification of viral RNA.** We mainly quantified viral RNA to measure the antiviral activity of drugs. Viral RNA was extracted with a QIAamp Viral RNA mini, RNeasy mini kit (QIAGEN), or MagMax Viral/Pathogen II Nucleic Acid Isolation kit (Thermo Fisher Scientific) and quantified by real time RT-PCR analysis with a one-step qRT-PCR kit (THUNDERBIRD Probe One-step qRT-PCR kit, TOYOBO) using 5'-ACAGGTACGTTAATAGTTAATAGCGT-3', 5'-ATATTGCACGACTACGCACACA-3', and 5'-FAM-ACACTAGCCATCTTACTGGCTTCG-TAMRA-3' (E-set) (Corman et al., 2020) or 5'-AAATTTGGGGAVVAGGAAC-3', 5'-TGGCAGCTGTGTAGGTCAAC-3', and 5'-FAM-ATGTCCGCATTGGCATGGA-TAMRA-3' (N2-set). Detection limit of SARS-CoV-2 RNA for N2-set was 38.1 cycle as C_t cycle.

**Detection of viral N protein.** Viral protein expression was detected using a rabbit anti-SARS-CoV N antibody (Mizutani et al., 2004) with AlexaFluor 568 anti-rabbit IgG or anti-rabbit IgG-HRP (Thermo Fisher) by indirect immunofluorescence or immunoblot analyses as previously reported (Ohashi et al., 2018).

**Cell viability and virus induced cytopathology.** Cell viability (Fig. 2B) was determined by MTT assay as previously reported (Ohashi et al., 2018). Virus-induced cytopathology was observed by microscopy at 48 h post-infection as previously reported (Matsuyama et al., 2020). Quantification of cell number (Fig. 1B) was performed with a high-content imaging system as shown below.

**Chemical screening.** We screened an FDA/EMA/PMDA-approved chemical library composed of 306 compounds by the following cytopathic effect assay to augment the throughput. Cells were treated with compounds at 8, 16, or 30 μM for 1 h during virus inoculation and for up to 72 h post-inoculation. The cells were then fixed with 4% paraformaldehyde and stained with DAPI to count viable cells using a high-content imaging system (ImageXpress Micro Confocal, Molecular Devices). Compounds that protected cells from virus-induced cytopathology and showed cell survival more than 20-fold of the control were selected as hits. Among 306 tested compounds, Cepharanthine, Lopinavir, Loteprednol, Nelfinavir, and Rapamycin were identified as hits. Lopinavir is currently being evaluated in clinical trials for treatment of COVID-19 (Cao et al., 2020). As Loteprednol and Rapamycin are steroid and immunosuppressant, respectively, which suppress immune response, we focused on Cepharanthine and Nelfinavir in this study.

**Virus-cell binding assay.** SARS-CoV-2 was preincubated with the indicated compounds at 37°C for 30 min. The SARS-CoV-2 (MOI=0.001) was exposed to VeroE6/TMPRSS2 cells at 4°C for varying time (5, 15, 30 and 50 min). After extensive wash, total RNA was extracted from cells and viral RNA was quantified by real time RT-PCR to measure cell-bound virus. In this assay, signal/noise ratio of detected RNA for the control with 30 min incubation (Fig. 4B) was more than 300-fold.
Docking simulation of compound binding with a target protein. The crystal structure of the main protease and spike protein were obtained from Protein Data Bank (6LU7 (Jin et al., 2020) and 6M0J (Lan et al., 2020)) and refined for docking simulations using the Protein Preparation Wizard Script within Maestro (Schrödinger, LLC). We carried out in silico library screening based on the active site pocket of the main protease using combined molecular docking with a protein-ligand interaction fingerprint scoring method against 8,085 known drugs obtained from the KEGG-Drug database (Kanehisa and Goto, 2000). For all compounds ionization and energy minimization were performed by the OPLS3 force field in the LigPrep Script of Maestro (Schrödinger, LLC). These minimized structures were used as input structures for docking simulations. Docking simulations were performed using the Glide (Friesner et al., 2004; Halgren et al., 2004) SP docking program (Schrödinger, LLC) with a grid box defined by N3 inhibitor molecule for main protease and ACE2 binding interface residues for spike protein using BioLuminate (Schrödinger, LLC).

In vitro SARS-CoV-2 protease assay. The SARS-CoV-2 encoded main protease was purchased from BPS Bioscience, Inc. (USA). The synthesized fluorogenic peptide Ac-Abu-Tle-Leu-Gln-MCA (Rut et al., 2020) was kindly provided by Peptide Institute, Inc. (Japan), and was used as a substrate for the proteolytic assay using the SARS-CoV-2 main protease. The assay was performed in the buffer (20 mM Tri-HCl, pH 7.3, 100 mM NaCl, 1 mM DTT, 1 mM EDTA) containing 20 µM of the fluorogenic peptide and 200 nM of the protease. The protease and NFV were pre-incubated at 37°C for 30 min. The reaction was initiated by addition of the substrate and incubated for 30 min at 37°C. The fluorescence of Aminomethylcoumarin due to cleavage of the fluorogenic peptide was monitored at 460 nm with excitation at 380 nm on a fluorescence plate reader (En Spire, Perkin Elmer).

Mathematical analysis. Determination of synergism between NFV and CEP and simulation of virus dynamics as well as the calculation of IIP are shown in detail in Supporting Note.

Statistics. Statistical significance estimated using the two-tailed Student’s t test (*p<0.05; **p<0.01; N.S., not significant).
Supplemental Note, related to Figure 2, 6, and 7.

Quantifying instantaneous inhibitory potential (IIP) from the dose-response curves of the drugs

The typical dose-response curves of a single antiviral drug can be analyzed using the following Hill function (Koizumi et al., 2017) (Fig. 2A):

\[ f_u = \frac{1}{1 + \left(\frac{D}{IC_{50}}\right)^m}. \]  

(1)

Here, \( f_u \) represents the fraction of infection events unaffected by the drug (i.e., \( 1 - f_u \) equals the fraction of drug-affected events). \( D \) is the drug concentration, \( IC_{50} \) is the drug concentration that achieves 50% inhibition of activity, and \( m \) is the slope of the dose-response curve (i.e., Hill coefficient) (Koizumi et al., 2017). Dose-response curves for drugs with higher \( m \) values show stronger antiviral activity at the same normalized drug concentration so long as the drug concentration is higher than \( IC_{50} \) (Fig. 2A). Least-square regression approach was used to fit Eq.(1) to dose-response data and estimate the values of \( IC_{50} \) and \( m \). Those estimated values for each drug against SARS-CoV-2 are summarized in Table S1.

Expected anti-SARS-CoV-2 effect of double-drug combinations by Bliss independence

We evaluated the effect of double-drug combinations for Bliss independence which is widely used to analyze drug combination data (Bliss, 1939; Kobayashi et al., 2014; Koizumi and Iwami, 2014; Tallarida, 2001). Bliss independence model assumes that each drug acts on different targets/mechanisms, and is defined as:

\[ f_u^{B\text{com}} = f_u^A(D) \times f_u^B(D), \]  

(3)

where \( f_u^{B\text{com}} \), \( f_u^A \) and \( f_u^B \) are the fractions of infection events unaffected by the combined drugs A (i.e., Nelfinavir: NFV) and B (i.e., Cepharanthine: CEP) expected by the Bliss model, single drug A and single drug B defined by Eq. (1), respectively. Using Eq. (2), we expected the anti-SARS-CoV2 effects of combined drugs A and B, \( 1 - f_u^{B\text{com}} \), from the anti-SARS-CoV-2 effects of the single drugs (Fig. S2).

However, the Bliss model ignores interactions in which drugs enhance each other effects. To address this point, we introduced the recent proposed model (Zimmer et al., 2016), called “dose model” considering the drug interactions, and further evaluated the expected antiviral effects (Fig. S2). This drug interaction is described by introducing interaction terms between drug pairs, that is, the “effective” concentration of drug A (i.e., NFV) and B (i.e., CEP), \( D_{A\text{com}} \) and \( D_{B\text{com}} \), are defined as follows;

\[ D_{A\text{com}} = D_A \left(1 + a_{AB} \frac{D_{B\text{com}}}{IC_{50}^B + D_{B\text{com}}}\right)^{-1}, \quad D_{B\text{com}} = D_B \left(1 + a_{BA} \frac{D_{A\text{com}}}{IC_{50}^A + D_{A\text{com}}}\right)^{-1}, \]

where \( D_A \) and \( D_B \) are the “true” concentrations, \( IC_{50}^A \) and \( IC_{50}^B \) are the concentrations that achieve 50% inhibition of activity, \( a_{AB} \) and \( a_{BA} \) are the interaction parameters for drug A and B, respectively. Note that \( IC_{50}^A \) and \( IC_{50}^B \) are corresponding to the estimations from the dose-response curves of a
single antiviral drug in combination treatment experiment, which is summarized in Table S1, and $a_{AB} = -0.462$ and $a_{BA} = 0.307$ are estimated from the dose-response curves of the double-drug combination. The dose model extended the Bliss model, thus, the expected anti-SARS-CoV-2 effect with effective concentration of drugs A and B (rather than the true concentrations) are calculated as $1 - f^{D\text{com}}_u(D)$ and

$$f^{D\text{com}}_u = f^A_u(D^\text{com}_A) \times f^B_u(D^\text{com}_B).$$

The dose model assumed that the effects of drugs on each other’s effective doses are multiplicative.

PK/PD/VD model for single- and double-drug combinations against SARS-CoV-2 infection

Based on a standard viral dynamics (VD) model (Ikeda et al., 2016), to describe COVID-19 dissemination among susceptible target cells, we used the following simple mathematical model proposed in (Kim et al., 2020):

$$\frac{df(t)}{dt} = -\beta f(t)V(t),$$

$$\frac{dV(t)}{dt} = \gamma f(t)V(t) - \delta V(t),$$

where $f(t)$ and $V(t)$ are the ratio of uninfected target cells and the amount of virus, respectively. The parameters $\beta$, $\gamma$, and $\delta$ represent the rate constant for virus infection, the maximum rate constant for viral replication and the death rate of infected cells, respectively. All viral load data including Singapore and Zhuhai patients (Young et al., 2020; Zou et al., 2020) were simultaneously fitted using a nonlinear mixed-effect modelling approach, which uses the whole samples to estimate population parameters while accounting for inter-individual variation. The estimated parameters and initial values used here are summarized in Table S3.

To investigate the expected outcome for anti-SARS-CoV-2 therapies with single-drug, we conducted in silico experiments with the following PK/PD/VD model for replication inhibitor such as Nelfinavir (Fig. 7);

$$\frac{df(t)}{dt} = -\beta f(t)V(t),$$

$$\frac{dV(t)}{dt} = (1 - \varepsilon(t) \times H(t))\gamma f(t)V(t) - \delta V(t),$$

and for entry inhibitor such as Cepharanthine;

$$\frac{df(t)}{dt} = -(1 - \eta(t) \times H(t))\beta f(t)V(t),$$

$$\frac{dV(t)}{dt} = (1 - \eta(t) \times H(t))\gamma f(t)V(t) - \delta V(t).$$

Here $H(t)$ is a Heaviside step function defined as $H(t) = 0$ if $t < T$: otherwise $H(t) = 1$, where $T$ is the initiation timing of the treatment, and the anti-SARS-CoV2 effect for $t > T$ are described as

$$\varepsilon(t) \text{ (or } \eta(t)\text{) } = 1 - f_u(D(t)) = 1 - \frac{1}{1 + \left(\frac{D(t)}{IC_{50}}\right)^m}, \quad D(t) = C_{\text{max}} e^{-kt}$$
where $C_{\text{max}}$ and $k$ are the peak drug concentration and the elimination rate for corresponding drug, respectively. The parameter values for each drug used here are summarized in Tables S1 and S2. Antiviral activities of CEP and NFV were calculated with the expected pharmacokinetics in human lung, based on the pharmacokinetics information for human peripheral blood and that for rat lung and peripheral blood for normalization (Yokoshima et al., 1986; Shetty et al., 1996; Ford et al., 2004).

For anti-SARS-CoV-2 therapies with double-drug combinations, we extended as the following PK/PD/VD model assuming the dose model;

\[
\frac{df(t)}{dt} = -\left(1 - \eta(t) \times H(t)\right)\beta f(t)V(t),
\]

\[
\frac{dV(t)}{dt} = (1 - \varepsilon(t) \times H(t))\left(1 - \eta(t) \times H(t)\right)\gamma f(t)V(t) - \delta V(t).
\]

Here $H(t)$ is a Heaviside step function defined as $H(t) = 0$ if $t < T$; otherwise $H(t) = 1$, and the anti-SARS-CoV2 effect are described as

\[
\varepsilon(t) = 1 - f_u^A(D_{\text{com}}^A(t)) = 1 - \frac{1}{1 + \left(\frac{D_{\text{com}}^A(t)}{I_C_{50}^A}\right)^{m_u^A}},
\]

\[
\eta(t) = 1 - f_u^B(D_{\text{com}}^B(t)) = 1 - \frac{1}{1 + \left(\frac{D_{\text{com}}^B(t)}{I_C_{50}^B}\right)^{m_u^B}},
\]

\[
D_{\text{com}}^A(t) = C_{\text{max}}^A e^{-k_A t} \left(1 + a_{AB} \frac{D_{\text{com}}^B(t)}{I_C_{50}^B + D_{\text{com}}^A(t)}\right)^{-1},
\]

\[
D_{\text{com}}^B(t) = C_{\text{max}}^B e^{-k_B t} \left(1 + a_{BA} \frac{D_{\text{com}}^A(t)}{I_C_{50}^A + D_{\text{com}}^B(t)}\right)^{-1}.
\]

Note that we here evaluated the double-drug combination of NFV and CEP (Fig. 7), and the pharmacokinetics of NFV and CEP, $D_{\text{com}}^A(t)$ and $D_{\text{com}}^B(t)$, under the combination, are different from those, $D_A(t)$ and $D_B(t)$, under the single-drug treatment because of the effective drug concentration.

**Evaluation of outcomes for anti-SARS-CoV-2 therapies**

The antiviral effect of the anti-viral therapy on SARS-CoV-2 dynamics using Eqs. (7-12) and our estimated parameter values was calculated (Fig. 7). We evaluated the outcomes for the therapies defined as “period until virus elimination” and “reduction of cumulative virus production” (Fig. S3). Note that the cumulative virus production, i.e., the area under the curve of viral load (AUC: $\int_0^{T_D} V(s) ds$), for SARS-CoV-2 was calculated, where $T_D$ is the time for SARS-CoV-2 achieved the detection limit.
Fig. S1. SARS-CoV-2 binding to cells was inhibited under CEP treatment, related to Figure 4. SARS-CoV-2 pretreated with DMSO (control) or CEP was exposed to VeroE6/TMPRSS2 cells at an MOI of 0.001 in the presence of DMSO (control) or CEP for 5, 15, 30, and 50 min at 4°C to allow virus-cell binding but not the following steps. After extensive wash, viral RNA was extracted from cells and was quantified by real time RT-PCR analysis. Viral RNA levels bound to cells were linearly increased along with the incubation time and CEP reduced the cell-bound viral RNA at any time points examined. This data was from three independent experiments (mean ± SD).
Fig. S2. Comparison of experimental data, Bliss model and Dose model for the double-drug combinations, related to Figure 6. Dose-response matrix of the double-drug combination (corresponding to Fig. 6A) are plotted in (A), and the expected anti-SARS-CoV-2 effects of the double-drug combination (NFV and CEP) by the Bliss model and Dose model are plotted in (B) and (C), respectively. Note that experimental measurements over 100% of viral RNA (implying large experimental variation because of small dose of antiviral drugs), colored by gray, were excluded in our analysis. The ratios of the values shown in (A) over those in (B) were calculated and are depicted in Fig. 6C in a 3D landscape. To increase the accuracy of evaluation, we employed 1.2-fold serial dilution of NFV from 2.24 μM and 1.6-fold serial dilution of CEP from 8.19 μM.
Fig. S3. Schematic representation of SARS-CoV-2 infection dynamics, related to Figure 7. A typical disease progress with viral load on patients undergoing therapy are shown. The outcomes for the therapies, that is, reduction in “period until virus elimination” and “cumulative virus production” are graphically depicted.
Supplemental Tables

Table S1. Estimated characteristic parameters of the tested antiviral drugs, related to Figure 2.

| Drug (unit)      | Class | IC₅₀         | m     |
|------------------|-------|--------------|-------|
| **Single-drug treatment** |       |              |       |
| Lopinavir (μM)   | RI    | 3.609        | 3.852 |
| Nelfinavir (μM)  | RI    | 0.765        | 5.079 |
| Favipiravir (μM) | RI    | 4.057 × 10⁻¹⁶¹ | 5.610 × 10⁻³ |
| Remdesivir (μM)  | RI    | 1.577        | 3.048 |
| Chloroquine (μM) | EI    | 1.313        | 1.984 |
| Cepharanthine (μM)| EI  | 0.351        | 2.307 |
| **Combination treatment** |   |            |       |
| Nelfinavir (μM)  | RI    | 1.317        | 4.043 |
| Cepharanthine (μM)| EI  | 0.991        | 3.174 |

RI, replication inhibitor; EI, entry inhibitor

IC₅₀, 50% inhibitory concentration
m, slope of the dose-response curve (i.e., Hill coefficient)
Table S2. Summary of pharmacokinetic parameters of anti-SARS-CoV-2 drugs, related to Figure 7.

| Parameter name        | Symbol | Unit     | Nelfinavir* | Cepharanthine** |
|-----------------------|--------|----------|-------------|-----------------|
| Single-compartment model |        |          |             |                 |
| Maximum concentration | $C_{\text{max}}$ | µM       | 9.32        | 12.3            | $3.49 \times 10^{-2}$ |
| Degradation rate      | $k$    | day$^{-1}$ | 4.89        | 0.318           |
| Dosing schedule       |        |          |             |                 |
| Initiation of treatment | $t^*$ | day      | 0.500       |                 |
| Dosing interval       | $\tau$ | day      | 0.333       | 7.00            | 1.00             |

Nelfinavir: 500 mg, TID, orally
Cepharanthine: 25mg, intravenous drip (i.v.) or 10 mg, oral administration (p.o.)

* Expected pharmacokinetics information in human lung. We estimated the scaling parameter of $C_{\text{max}}$ between lung and peripheral blood in rats (Shetty et al., 1996). We then calculated $C_{\text{max}}$ in human lung based on that in human peripheral blood assuming the scaling parameter is the same between humans and rats. Since the half-life of NFV in the cells was reported to be almost the same as that in plasma (Ford et al., 2004), we used the information for the half-life of NFV in plasma for that in the lung.

** Expected pharmacokinetics information in human lung. We estimated the degradation rate, $k$, in rat lung (Yokoshima et al., 1986), and the scaling parameter of $C_{\text{max}}$ between lung and peripheral blood in rat. Then we used the degradation rate for human lung and calculated $C_{\text{max}}$ in human lung by that in human peripheral blood assuming the scaling parameter is same between humans and rats.
Table S3. Estimated population parameters and initial values for SARS-CoV-2 infection, related to Figure 7.

| Parameter name                        | Symbol | Unit          | Value           |
|---------------------------------------|--------|---------------|-----------------|
| Maximum rate constant for viral replication | $\gamma$  | day$^{-1}$ | 3.16            |
| Rate constant for virus infection      | $\beta$ | (copies/ml)$^{-1}$ day$^{-1}$ | $9.77 \times 10^{-6}$ |
| Death rate of infected cells           | $\delta$ | day$^{-1}$ | 0.615           |
| Initial viral load                    | $V(0)$ | copies/ml    | $5.64 \times 10^3$ |
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