Discovery of M Protease Inhibitors Encoded by SARS-CoV-2

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ABSTRACT The coronavirus (CoV) disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome CoV-2 (SARS-CoV-2) is a health threat worldwide. Viral main protease (Mpro, also called 3C-like protease [3CLpro]) is a therapeutic target for drug discovery. Herein, we report that GC376, a broad-spectrum inhibitor targeting Mpro in the picornavirus-like supercluster, is a potent inhibitor for the Mpro encoded by SARS-CoV-2, with a half-maximum inhibitory concentration (IC50) of 26.4 ± 1.1 nM. In this study, we also show that GC376 inhibits SARS-CoV-2 replication with a half-maximum effective concentration (EC50) of 0.91 ± 0.03 μM. Only a small portion of SARS-CoV-2 Mpro was covalently modified in the excess of GC376 as evaluated by mass spectrometry analysis, indicating that improved inhibitors are needed. Subsequently, molecular docking analysis revealed that the recognition and binding groups of GC376 within the active site of SARS-CoV-2 Mpro provide important new information for the optimization of GC376. Given that sufficient safety and efficacy data are available for GC376 as an investigational veterinary drug, expedited development of GC376, or its optimized analogues, for treatment of SARS-CoV-2 infection in human is recommended.

KEYWORDS COVID-19, SARS-CoV-2, Mpro, antiviral research, GC376, M protease

Coronavirus (CoV) infection in humans and other animals has resulted in a variety of highly prevalent and serious diseases, including severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). Since late 2019, novel coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome CoV-2 (SARS-CoV-2), has spread from Wuhan City in China to the whole world (1, 2). As with SARS-CoV and MERS-CoV, the newly identified SARS-CoV-2 belongs to the genus Betacoronavirus and has a zoonotic origin (3, 4). SARS-CoV-2 causes common symptoms, including fever, cough, and shortness of breath. Complications may include pneumonia and acute respiratory distress syndrome (5, 6).

The genome of COVID-19 virus consists of about 30,000 nucleotides; its replicase gene encodes two overlapping polyproteins, pp1a and pp1ab, which are needed for virus replication and transcription. The functional viral proteins are released from the polypeptide through proteolysis, mainly by the main protease (Mpro), which is also referred as 3C-like protease (7). Mpro can digest at least 11 conserved sites within viral polyproteins. Viral Mpro has been considered a therapeutic target for the development

Citation Hung H-C, Ke Y-Y, Huang S-Y, Huang P-N, Kung Y-A, Chang T-Y, Yan K-J, Peng T-T, Chang S-E, Huang C-T, Tsai Y-R, Wu S-H, Lee S-J, Lin J-H, Liu B-S, Sung W-C, Shih S-R, Chen C-T, Hsu JT-A. 2020. Discovery of M protease inhibitors encoded by SARS-CoV-2. Antimicrob Agents Chemother 64:e00872-20. https://doi.org/10.1128/AAC.00872-20.

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Received 4 May 2020
Returned for modification 15 June 2020
Accepted 9 July 2020
Accepted manuscript posted online 15 July 2020
Published 20 August 2020

September 2020 Volume 64 Issue 9 e00872-20 Antimicrobial Agents and Chemotherapy aac.asm.org 1
of an effective antiviral treatment (8). Among all the mature structural or nonstructural proteins in SARS-CoV-2, Mpro is the most conserved target region within the whole viral genome (9). Due to the severity of SARS-CoV-2 infection, it is important to emphasize drug discovery for SARS-CoV-2 based on existing drugs for immediate uses or an expedited development timeline.

We previously discovered several small-molecule inhibitors for SARS-CoV during the SARS outbreak in 2003 (10). The Mpro encoded by SARS-CoV-2 represents a key target for anti-SARS-CoV-2 strategies. However, to date, a promising SARS-CoV-2 Mpro protease inhibitor has been lacking. Herein, we establish the SARS-CoV-2 Mpro protease fluorescence-based assay to screen for potential inhibitors. Furthermore, molecular modeling studies were carried out to further demonstrate the interaction of Mpro with GC376. GC376 or its optimized analogues hold great promise to be developed in humans with SARS-CoV-2 infection, alone or together with other antiviral drugs.

RESULTS

Fluorescence resonance energy transfer (FRET)-based screening assays. The Mprotease (Mpro) encoded by the SARS and SARS-CoV-2 coronaviruses differ in only 12 amino acid residues. According to our previous experience during the SARS outbreak, SARS-CoV-2 Mpro is expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* (11, 12). The GST fusion protein was purified by glutathione affinity chromatography. The fusion protein was cleaved by factor Xa, resulting in the generation of a prominent protein band with an apparent molecular mass of 35 kDa, the mature SARS-CoV-2 Mpro (see Fig. S1c in the supplemental material).

Purified Mpro proteins were checked for the proteolytic activity that cleaves the EDANS-KTSAVLQSGFRKME-DABCYL substrate, where EDANS is 5-((2-aminoethyl)amino) naphthalene-1-sulfonic acid and DABCYL is 4-(dimethylaminoazo)benzene-4-carboxylic acid (Fig. S2a). The purified enzyme was assayed using the FRET technique as described in Materials and Methods. The performance of the FRET assay was assessed, and the signal-to-noise ratio was determined to be >20 (Fig. S2b). The Z-factor value for the assay was 0.9, which corresponds to a valid screening system. The Mpro of feline infectious peritonitis virus (FIPV) was prepared similarly. The same fluorogenic substrate, EDANS-KTSAVLGFRKME-DABCYL, was equally applicable in activity assessments for FIPV Mpro.

Inhibitory activities of SARS-CoV-2 Mpro by the Zinc ion, GC376, and lopinavir. In this study, based on the SARS-CoV-2 Mpro activity assay, we screened a collection of protease inhibitors, such as reported Mpro inhibitors for relevant viruses and clinically approved human immunodeficiency virus (HIV) protease inhibitors. At 10 μM, GC376 (Fig. 1A), a broad-spectrum antiviral protease inhibitor used to treat cats with FIPV infection (13), showed complete inhibition of SARS-CoV-2 Mpro activity. Since GC376 was well characterized for its inhibition of Mpro encoded by FIPV, we conducted a head-to-head comparison for the inhibitory activity of GC376 on SARS-CoV-2 Mpro and FIPV Mpro. SARS-CoV-2 and SARS-CoV share high identity in amino acid sequence (Fig. S3a), whereas SARS-CoV-2 Mpro and FIPV Mpro share 45% identity in amino acid sequence (Fig. S3b). These two viral proteases also share similar folding and crystal structures (14, 15). In this study, we found that GC376 is an extremely potent inhibitor of the Mpro encoded by SARS-CoV-2, with a half-maximum inhibitory concentration (IC$_{50}$) of 26.4 ± 1.1 nM (Fig. 1B). Subsequent analysis showed that GC376 is a competitive inhibitor of the Mpro from SARS-CoV-2, with a binding constant (K$_{i}$) of 12 ± 1.4 nM (Fig. 1C). In contrast, the IC$_{50}$ and K$_{i}$ of GC376 toward FIPV Mpro are 118.9 ± 1.1 nM and 42.5 ± 2.9 nM, respectively (Fig. S4a and b).

To examine whether a covalent adduct is formed, SARS-CoV-2 Mpro incubated with GC376 was subject to mass spectrometry (MS) analysis in accordance with a method described previously. Indeed, a gain of 403.2 Da in mass was observed in new peaks from GC376-incubated SARS-CoV-2 Mpro, indicating the same mechanism for adduct formation as described previously (13; also results not shown). Through the mass spectrometry analysis, we observed a new MS peak with a mass value of 34,194.0 Da,
which is equal to the dihydrogen molecular weight of M protease (33,790.8), reflecting conjugation with only one GC376 molecule. Even in an excess of GC376, only 30% of the Mpro enzyme was conjugated based on the peak intensity. With the X-ray (NCBI Protein Data Bank accession number 7BRR; release date, 13 May 2020) and the MS analyses performed in this study, it is evident that GC376 forms a covalent bond with Cys145 of Mpro. That only a small portion of SARS-CoV-2 Mpro was covalently modified in a 25:1 molar excess of GC376 indicates that improved inhibitors are needed.

All the HIV protease inhibitors, including lopinavir, ritonavir, fosamprenavir, saquinavir, nelfinavir, atazanavir, darunavir, amprenavir, tipranavir, and indinavir, showed no inhibitory activity at 20 \( \mu \text{M} \), reflecting the fact that no benefit was observed with lopinavir-ritonavir treatment in patients with severe COVID-19 (16). Since \( \text{Zn}^{2+} \) was shown to inhibit 3CLpro encoded by SARS-CoV (17), ZnCl\(_2\) and ZnSO\(_4\) were evaluated for their activity against SARS-CoV-2 Mpro in this study. Zinc salts have been shown to

![Graph showing the inhibitory activity of GC376 against SARS-CoV-2 Mpro with IC\(_{50}\) and Ki values.](image-url)

**FIG 1** Structure of GC376 and the IC\(_{50}\) and the inhibitory constant (K\(_i\)) of recombinant Mpro of SARS-CoV-2. (A) GC376 is a peptidomimetic antiviral drug. The IC\(_{50}\) (B) and the K\(_i\) (C) of Mpro of SARS-CoV-2 are shown. The proteolytic activity of Mpro was determined by the FRET protease assay, as described in the text. RFU, relative fluorescence units.
completely inhibit the activity of SARS-CoV-2 Mpro at the micromolar level (data not shown).

**Antiviral effects of GC376 on the replication of SARS-CoV-2 in cell culture.** To confirm that GC376 inhibited SARS-CoV-2 replication and cellular toxicity in cell culture, GC376 was tested for inhibition of SARS-CoV-2 infection in Vero E6 cells with 100 TCID<sub>50</sub> per well in 96-well plates. SARS-CoV-2-infected cells were treated with increasing concentrations of GC376, and protection from cytopathic effects (CPE) was visually observed. GC376 dose dependently showed a reduction of the viral CPE (Fig. S5). After the cells were stained with crystal violet, their optical density at 570 nm (OD<sub>570</sub>) was measured (Fig. 2A). The results showed that GC376 inhibited SARS-CoV-2 infection, with an EC<sub>50</sub> of 0.91 ± 0.03 μM (Fig. 2B). GC376 exhibited a broad-spectrum antiviral activity against several coronaviruses in various cell lines (13). To evaluate whether GC376 was cytotoxic to cells, Vero E6 cells were treated with different concentrations of GC376 up to 100 μM, and cell viability was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. We found that GC376 did not show cytotoxicity in Vero E6 cells up to
100 μM (Fig. 2B). Hence, we concluded that the selectivity index (SI) of GC376 was >114.

**Molecular docking.** To inform lead optimization efforts starting from GC376, in silico calculations to correlate IC₅₀ and Kᵢ into binding energy between GC376 and Mₚᵣᵒ were attempted in accordance with, in part, our previous work (18–20). By our calculation, the free binding energies of GC376 with SARS-CoV-2 Mₚᵣᵒ and FIPV Mₚᵣᵒ are −51.59 kcal/mol and −32.42 kcal/mol, respectively. As shown in Fig. 3A and B, upon removal of the bisulfite group, the compound is converted to an aldehyde form, giving rise to a covalent bond with catalytic Cys145. This result is in congruence with the cocrystal structures of GC376 and MERS Mₚᵣᵒ, where GC376 forms a covalent bond with Cys148 (31). In Fig. 3C, the amino acid residues on the inner surface of the substrate binding sites within FIPV Mₚᵣᵒ and SARS-CoV-2 Mₚᵣᵒ are well conserved. Only two sites of amino acid residues are different between the two Mₚᵣᵒ binding pockets. In SARS-CoV-2, the Gln189 on the surface of the binding pocket of SARS-CoV-2 Mₚᵣᵒ supports a H bond with the carbamate moiety of GC376. In contrast, this H bond cannot be formed because the counterpart residue in FIPV Mₚᵣᵒ is Pro188, rather than Gln. It appears that due to the covalent binding with Cys145 and hydrogen binding with Gln189 within the substrate binding pocket of SARS-CoV-2 Mₚᵣᵒ, GC376 was induced to bind more snugly into the pocket through a strong H bond network with Phe140, Gly143, Ser144, Cys145, His163, His164, Glu166, and Gln189 (Fig. 3A). In contrast, GC376 forms only a weaker H bond network with Gly142, His162, and Glu165 (Fig. 3B). The other different sites are Ser144 in SARS-CoV-2 Mₚᵣᵒ and Thr143 in FIPV Mₚᵣᵒ. These differences had little influence on the binding network. The root mean square deviation (RMSD) between the two docking conformations of GC376/SARS-CoV-2 and GC376/FIPV is 1.16 Å. Importantly, our in silico prediction has informed our potential direction to improve GC376 with respect to its potency and further drug-like properties. With the docking analyses, GC376 may be improved by replacing the benzene group with H bond donors to interact with Glu166. The other alternative for improvement of binding potency is to replace the isobutyl group with moieties of a less bulky hydrophobic group so as to form interactions with Met49 (Fig. 3C). R. J. Hussey et al. have reported that the Michael acceptor inhibitor, acetyl-Glu-Phe-Gln-Leu-Gln-CH=CHCOO−, forms a covalent bond with catalytic Cys139 in norovirus 3CLₚᵣᵒ (22), suggesting an alternative avenue for optimization of GC376. After the submission of this paper, the crystal structure of the 3CL protease complexed with GC376 (accession no. 7BRR; release date, 13 May 2020) became available in the NCBI Protein Data Bank. When we compared the cocrystal X ray with our docked Mₚᵣᵒ-GC376 model based on 6LU7, the RMSD was 0.74, indicating that the in silico docking approach employed in this study adequately predicted the real complexed structure before its availability. In Fig. S6, the modeled conformation is shown in green and aligned to the X-ray result in pink.

**DISCUSSION**

To date, no proven effective therapy has been shown to be effective for SARS-CoV-2 infection (9). As of the submission date of this paper, the once-promising medicines, including remdesivir and hydroxychloroquine, are facing challenges after more stringently controlled observations and trials (23, 24). When coronaviruses replicated inside cells, cellular innate immunity was shown to be compromised by Mₚᵣᵒ (25). We have previously shown that the compromised interferon (IFN)-mediated antiviral mechanism of viral 3Cₚᵣᵒ of enterovirus 71 can be rescued by effective protease inhibitor (26). Thus, effective inhibition of viral protease may not only restrict virus replication but also prevent interruption of the antiviral IFN pathway.

We also found that GC376 is a promising Mₚᵣᵒ inhibitor for SARS-CoV-2. GC376 is a dipeptidyl bisulfite adduct salt with excellent inhibitory activity against several picornaviruses and coronaviruses (13, 27, 28). Administration of GC376 leads to a full recovery in laboratory cats with FIPV infection, a highly fatal feline disease (29). Y. Kim in 2016 also studied the pharmacokinetic properties and the safety of GC376 in laboratory cats. In their safety study of GC376, no adverse effects were observed and no
Docked conformations of GC376 in SARS-CoV-2 and FIPV M\textsuperscript{pro} proteases. (A) GC376 docking to the SARS-CoV-2 M\textsuperscript{pro} protein X-ray structure (NCBI Protein Data Bank accession no. 6LU7). (B) GC376 docking to the FIPV 3C-like protease protein X-ray structure (accession no. 4ZRO). (C) Structure alignment of FIPV (accession no. 4ZRO, purple) and SARS-CoV-2 (accession no. 6LU7, white). The docking result of GC376 in FIPV displays as cyan, and the docking result of GC376 displays as orange. The red box shows the different residues in the FIPV and SARS-CoV-2 binding sites.
changes in clinical lab parameters were reported in cats subcutaneously given GC376 at 10 mg/kg of body weight/dose twice a day for 4 weeks (29). In this safety study, the plasma drug concentrations were shown to remain slightly above 1,000 ng/ml (i.e., ~2,000 nM, as the molecular weight of GC376 is 507.53), which was well above the concentrations needed for effective inhibition of SARS-CoV-2 as observed in this study. Therefore, the existing pharmacology and efficacy data for GC376 as an investigational drug in cats with FIPV infection encourage a proof-of-principle study of COVID-19 patients and then of the in vitro and in vivo antiviral activities of GC376 or further-optimized analogues.

MATERIALS AND METHODS

Drugs and reagents. The test compounds were mainly from Selleck and MedChemExpress. Several in-house-collected compounds, including HIV protease inhibitors, GC376, and natural products, were also screened as Mpro inhibitors. GC376 was purchased from Biosynth Carbosynth. It was dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock solution and stored at −20°C. The fluorogenic peptide substrate DABCYL-KTSAVLQSGFRKME-EDANS utilized in the fluorescence resonance energy transfer (FRET) assay for Mpro was obtained from Genesis Biotechnology Inc.

Expression and purification of 3C-like proteases (Mpro). To express the M proteases from SARS-CoV-2 and FIPV, CDNs encoding factor Xa cleavage site and the genes as deduced from the Wuhan-Hu-1 strain (GenBank accession no. NC_045512.2) and WSU-79/1146 strain (NCBI accession no. AAY32595.1) were optimized for codon preference in E. coli, respectively. The amino acid sequence of SARS-CoV-2 Mpro is shown in Fig. S1a in the supplemental material. The synthetic cDNA (Bio Basic, Canada) encoding factor Xa recognition site and Mpro was inserted into the expression plasmid vector pGEX-4T-1 (GE Health Care) using BamHI and XhoI restriction enzyme cutting sites (Fig. S1b); an ampicillin resistance gene was used as a selection marker.

The recombinant plasmid was transfected into the Rosetta 2(DE3)pLysS strain (Novagen) and an E. coli host, and the overnight culture in LB medium was refreshed to an OD600 of 0.8 at 37°C and then induced with 1 mM IPTG for 5 h at 25°C. The cells were harvested by centrifugation at 4°C (6,000 rpm, 10 min) followed by sonication in lysis buffer containing 10 mM phosphate, 2.7 mM KCl, pH 7.4, 0.1% Triton X-100. The GST-Mpro fusion protease was purified by glutathione Sepharose 4 Fast Flow (GE Healthcare) with purification buffer (50 mM Tris, pH 8.0, 10 mM glutathione). The purified GST-Mpro fusion protease was changed to factor Xa digestion buffer (20 mM HEPES, pH 6.0, 0.4 mM EDTA, 1 mM dithiothreitol [DTT], 1% glycerol). The FRET substrate, DABCYL-KTSAVLQSGFRKME-EDANS, was then added at a final concentration of 10 μM to the enzymatic reaction mixture for 30 min at RT. The readouts for the same compound concentrations with the substrate without Mpro enzyme were measured as a blank. The fluorescence signals (excitation/emission, 355 nm/460 nm) of released EDANS were measured (excitation/emission, 355 nm/460 nm) of released EDANS were measured using a fluorometer (Victor2; PerkinElmer). The results were plotted as dose inhibition curves using nonlinear regression with a variable slope to determine the IC50 values of inhibitor compounds (with nonlinear regression of competitive enzyme kinetics).

Protease activity assay. The protease assays were performed in 96-well, black, flat-bottomed microtiter plates (Greiner Bio-One, Germany) with a final volume of 100 μl. SARS-CoV-2 Mpro recombinant protease, at a final concentration of 20 nM, was preincubated for 10 min at room temperature (RT) with compounds at different concentrations in the assay buffer (20 mM HEPES, pH 6.0, 0.4 mM EDTA, 1 mM dithiothreitol [DTT], 1% glycerol). The FRET substrate, DABCYL-KTSAVLQSGFRKME-EDANS, was then added at a final concentration of 10 μM to the enzymatic reaction mixture for 30 min at RT. The readouts for the same compound concentrations with the substrate without Mpro enzyme were measured as a blank. The fluorescence signals (excitation/emission, 355 nm/460 nm) of released EDANS were measured using a fluorometer (Victor2; PerkinElmer). The results were plotted as dose inhibition curves using nonlinear regression with a variable slope to determine the IC50 values of inhibitor compounds (with nonlinear regression of competitive enzyme kinetics).

Antiviral assay and cytotoxicity assay. To examine the anti-SARS-CoV-2 activity of positive compounds identified in the Mpro activity assay, TCID50 (50% tissue culture infectious doses) were determined using 2-fold serial dilutions of hit compounds starting from 50 μM. In brief, each well of a 96-well tissue culture plate was seeded with 200 μl of a 1.15 × 104 Vero E6 cells/ml in minimal essential medium (MEM) with 10% fetal bovine serum (FBS). After cells were incubated for 18 to 24 h at 37°C, SARS-CoV-2/human/ TWN/CGMH-CGU-01/2020 virus was added at 100 TCID50 per well mixed with different concentrations of GC376. After 5 days, cells were fixed with formaldehyde and stained with 0.1% crystal violet as a selection marker.

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Protease activity assay. The protease assays were performed in 96-well, black, flat-bottomed microtiter plates (Greiner Bio-One, Germany) with a final volume of 100 μl. SARS-CoV-2 Mpro recombinant protease, at a final concentration of 20 nM, was preincubated for 10 min at room temperature (RT) with compounds at different concentrations in the assay buffer (20 mM HEPES, pH 6.0, 0.4 mM EDTA, 1 mM dithiothreitol [DTT], 1% glycerol). The FRET substrate, DABCYL-KTSAVLQSGFRKME-EDANS, was then added at a final concentration of 10 μM to the enzymatic reaction mixture for 30 min at RT. The readouts for the same compound concentrations with the substrate without Mpro enzyme were measured as a blank. The fluorescence signals (excitation/emission, 355 nm/460 nm) of released EDANS were measured using a fluorometer (Victor2; PerkinElmer). The results were plotted as dose inhibition curves using nonlinear regression with a variable slope to determine the IC50 values of inhibitor compounds (with GraphPad Prism 5.0). IC50 measurements were performed with various substrate concentrations of 5, 10, 20, and 40 μM and with a range of inhibitor concentrations (0, 8, 40, 200, and 1,000 nM) in a reaction mixture containing 20 nM Mpro at 37°C for 30 min. The IC50 value was computed using GraphPad Prism 5.0 software by nonlinear regression of competitive enzyme kinetics.

Antiviral assay and cytotoxicity assay. To examine the anti-SARS-CoV-2 activity of positive compounds identified in the Mpro activity assay, TCID50 (50% tissue culture infectious doses) were determined using 2-fold serial dilutions of hit compounds starting from 50 μM. In brief, each well of a 96-well tissue culture plate was seeded with 200 μl of a 1.15 × 104 Vero E6 cells/ml in minimal essential medium (MEM) with 10% fetal bovine serum (FBS). After cells were incubated for 18 to 24 h at 37°C, SARS-CoV-2/human/TWN/CGMH-CGU-01/2020 virus was added at 100× TCID50 per well mixed with different concentrations of GC376. After 5 days, cells were fixed with formaldehyde and stained with 0.1% crystal violet as described previously (21). The concentration required for the tested compound to reduce the cytopathic effects (CPE) of the virus by 50% (the 50% effective concentration [EC50]) was determined. The IC50 was calculated using GraphPad Prism 6 to assess the inhibition percentage at different inhibitor concentrations. To estimate the safety profile, an in vitro cytotoxicity study of GC376 was performed. We used the MTT assay to investigate the cytotoxicities of these compounds on Vero E6 cells. The half of the cytotoxic concentration (CC50) values were calculated from the percentages of cells whose viability was inhibited by GC376 at various concentrations.

Molecular modeling. The docking of compounds to the binding sites of SARS-CoV-2 Mpro (NCBI Protein Data Bank accession no. 6LU7) (14) or Mpro of feline infectious peritonitis virus, FIPV Mpro (NCBI Protein Data Bank accession no. 4ZRO) (6), was explored using the BIOVIA 2018/LigandFit program.
(BIOVIA, Inc., San Diego, CA). The detailed method of LigandFit has been described previously (30). To illustrate the binding interactions, GC376 was docked to the binding site. The binding pocket was identified from the MERS and GC376 cocrystal structures (NCBI Protein Data Bank accession no. SWKJ (31). The force field for calculating ligand-receptor interaction energies employed the piecewise linear potential 1 (PLP1). The rectangular grid was set at 0.5 Å spacing, and the extension from the site was set as 8 Å. The number of docking poses was set as 50 with default parameters. The docking root mean square (RMS) threshold for ligand site matching was set as 5 Å. The method of steepest descent for rigid-body minimization during pose docking was used. The covalent docking calculation was performed using the two-point attractor method by the AutoDock Tools (version 1.5.6) as described previously (17). The decision of the best pose was based on the similar conformations of the MERS complex cocrystal structure.

Molecular weight analysis using MS. The premixed GC376 compound (1 μl, 10 mM in DMSO) and SARS-CoV-2 Mpro (5 μl, 2.5 mg/ml) were incubated at 25°C for 30 min. Subsequently, 10 μl of the reaction mixture was transferred into 40 μl of the infusion solution (50% acetonitrile in 0.1% formic acid) for measuring the molecular weight using a quadrupole time of flight (QTOF) mass spectrometer (G1; Waters) through the direct infusion model. The ion signal (m/z) was acquired in positive-ion mode with a capillary temperature of 100°C and an electrospray voltage of 2,800 V in the scan range from 800 to 2,500 m/z. Mass deconvolution was performed using Waters MassLynx (v4.1) software using the MaxEnt 1 program with a half-height of 0.1 Da and a maximum number of interactions of 100.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

ACKNOWLEDGMENTS
This work was financially supported by a special government fund to tackle the COVID-19 pandemic from the Ministry of Health and Welfare, Taiwan, and a 5-year grant for the Research Center for Emerging Viral Infections (Chang Gung University) from the Featured Areas Research Center Program within the framework of the Higher Education Sprot Project by the Ministry of Education (MOE) and the Ministry of Science and Technology (MOST) in Taiwan.

The team also thanks Huai-Tzu Chang for coordination and facilitation on the project.

We declare that we have no competing interests.

REFERENCES
1. Wu D, Wu T, Liu Q, Yang Z. 12 March 2020. The SARS-CoV-2 outbreak: what we know. Int J Infect Dis https://doi.org/10.1016/j.ijid.2020.03.004.
2. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. 2020. The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol 5:536–544. https://doi.org/10.1038/s41556-020-0695-x.
3. Hu B, Ge X, Wang LF, Shi Z. 2015. Bat origin of human coronaviruses. Viriol J 12:221. https://doi.org/10.1186/s12985-015-0422-1.
4. Zhou P, Wang X, Zhang XS, Wu Y, Li Y, Tong S, Ren R, Shi D, Wang H, Lou X, Feng Z, Zhao JZ, Liu H, Jiang S, Xiao Y, Dai J, Hu B, Zhou Y, Liu H, Zhang X, Liu Q, Wu J, Wang Q, Zhang M, Yang H, Liu SY, Zhu T, Zhang D, Hu Y, Wang H, Jin Y, Fang F, Wang X, Gao S, Yang L, Wei W, Shi D, Shi Z. 30 January 2020. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 579: 270–273. https://doi.org/10.1038/s41586-020-2012-7.
5. Wolff R, Corman VM, Guggemos W, Seilmaier M, Zange S, Muller MA, Niemeyer D, Jones TC, Vollmar P, Rothe C, Hoeniger M, Luch M, Bierer B, Allam MG, Schild H, Muench H, Hengartner J, Eichler P, Chuchro M, Boltanski M, Stackebrandt E, Wittmann T, Wirtz S, Drosten C. 2020. Evaluation of metal-conjugated compounds as inhibitors of 3CLpro of SARS-CoV-2. FEBS Lett 594:146–152. https://doi.org/10.1002/2098-2763.e2020).
6. WHO. 2020. Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19). World Health Organization, Geneva, Switzerland. https://www.who.int/docs/default-source/coronaviruse/who-china -joint-mission-on-covid-19-final-report.pdf.
7. Anand K, Palm GJ, Mesters JR, Siddell SG, Ziebuhr J, Hilgenfeld R. 2002. Structure of coronavirus main proteinase reveals combination of a chymotrypsin fold with an extra alpha-helical domain. EMBO J 21:3123–3124. https://doi.org/10.1093/emboj/cdf327.
8. Sisay M. 2020. 3CLpro inhibitors as a potential therapeutic option for COVID-19: available evidence and ongoing clinical trials. Pharmacol Res 156:104779. https://doi.org/10.1016/j.phrs.2020.104779.
9. Liu C, Zhou Q, Li Y, Garner LV, Watkins SP, Carter LJ, Smoot J, Gregg AC, Daniels AD, Jervey S, Albaiu D. 2020. Research and development on therapeutic agents and vaccines for COVID-19 and related human coronavirus diseases. ACS Cent Sci 6:315–331. https://doi.org/10.1021/acscentsci.0c00272.
10. Wu CJ, Jan JT, Chen CM, Hsieh HP, Hwang DR, Liu HW, Liu CY, Huang HW, Chen SC, Hong CF, Lin RK, Chao YS, Hsu JT. 2004. Inhibition of severe acute respiratory syndrome coronavirus replication by niclosamide. Antimicrob Agents Chemother 48:2693–2696. https://doi.org/10.1128/AAC.48.7.2693-2696.2004.
11. Kuo CJ, Chi YH, Hsu JT, Liang PH. 2004. Characterization of SARS main protease and inhibitor assay using a fluorogenic substrate. Biochem Biophys Res Commun 318:862–867. https://doi.org/10.1016/j.bbrc.2004.08.015.
12. Wu CJ, Kuo CJ, Hsieh HP, Wang YC, Huang KK, Lin CP, Huang PF, Chen X, Liang PH. 2004. Evaluation of metal-conjugated compounds as inhibitors of 3CL propeptase of SARS-CoV. FEBS Lett 574:116–120. https://doi.org/10.1016/j.febslet.2004.07.018.
13. Kim Y, Lovell S, Tiew KC, Mandapadu SR, Alliston KR, Batalle KP, Groutas WC, Chang KO. 2012. Broad-spectrum antivirals against 3C or 3C-like proteases of picornaviruses, noroviruses, and coronaviruses. J Virol 86:11754–11762. https://doi.org/10.1128/JVI.01348-12.
14. Hatada R, Okuwa K, Motozuki Y, Handa Y, Suzuki K, Komeiji Y, Okiyama Y, Tanaka S. 15 June 2020. Fragment molecular orbital based interaction analyses on COVID-19 main protease—inhibitor N3 complex (PDB ID: 6LU7). J Chem Inf Model https://doi.org/10.1021/acs.jcim .0c00283.
15. St John SE, Therkelsen MD, Nylapatla PR, Osswald HL, Ghosh AK, Mecsek AD. 2015. X-ray structure and inhibition of the feline infectious peritonitis virus 3C-like protease: structural implications for drug design. Bioorg Med Chem Lett 25:5072–5077. https://doi.org/10.1016/j.bmcl .2015.10.023.
16. Cao B, Wang Y, Wen D, Liu W, Wang J, Fan G, Ruan L, Song B, Cai Y, Wei M, Li X, Xia J, Chen N, Xiang J, Yu T, Bai T, Xie X, Zhang L, Li C, Yuan Y, Chen H, Li H, Huang H, Tu S, Feng F, Li Y, Wei Y, Dong X, Qu Z, Lu S, Hu X, Ruan S, Luo S, Wu J, Peng L, Cheng F, Pan L, Zou J, Jia C, Wang J, Liu X, Wang S, Wu X, Ge Q, He J, Zhan H, Qiu F, Guo L, Huang C, Jaki T, Hayden FG, Horby PW, Zhang D, Wang C. 2020. A trial of lopinavir-ritonavir in adults hospitalized with severe Covid-19. N Engl J Med 382:1787–1799. https://doi.org/10.1056/NEJMoa2001282.

17. Gawehn E, Hiss JA, Schneider G. 2016. Deep learning in drug discovery. Mol Inform 35:3–14. https://doi.org/10.1002/minf.201501008.

18. Hsieh HP, Wang WC, Shiao HY, Ke YY, Lin WH, Hsu JTA, Chen CT, Yeh TK, Hung HC, Tseng CP, Yang JM, Ju YW, Tseng SN, Chen YF, Chao YS, Hsieh HP, Shih SR, Hsu JT. 2011. Optimization of ligand and lipophilic efficiency to identify an in vivo active furano-pyrimidine aurora kinase inhibitor. J Med Chem 54:5247–5260. https://doi.org/10.1021/jm4006059.

19. Shiao HY, Coumar MS, Chang CW, Ke YY, Chi YH, Chu CY, Sun HY, Chen CH, Lin WH, Fung KS, Kuo PC, Huang CT, Chang KY, Lu CT, Hsu JTA, Chen CT, Jiaang WT, Chao YS, Hsieh HP. 2013. Aurorainhibitors for the treatment of AML and solid tumors: rational design, synthesis, in vitro and in vivo evaluation. Absstr Pap Am Chem Soc 253.

20. Cournia Z, Allen B, Sherman W. 2017. Relative binding free energy calculations in drug discovery: recent advances and practical considerations. J Chem Inf Model 57:2911–2937. https://doi.org/10.1021/acs.jcim.7b00564.

21. Hung HC, Tseng CP, Yang JM, Ju YW, Tseng SN, Chen YF, Chao YS, Hsieh HP, Shih SR, Hsu JT. 2009. Aurintricarboxylic acid inhibits influenza virus neuraminidase. Antiviral Res 81:123–131. https://doi.org/10.1016/j.antiviral.2008.10.006.

22. Hussey RJ, Coates L, Gill RS, Erskine PT, Coker SF, Mitchell E, Cooper JB, Wood S, Broadbridge R, Clarke IN, Lamden PR, Shoolingin-Jordan PM. 2011. A structural study of norovirus 3C protease specificity: binding of a designed active site-directed peptide inhibitor. Biochemistry 50:240–249. https://doi.org/10.1021/bi1008497.

23. Colson P, Rolain JM, Raoult D. 2020. Chloroquine for the 2019 novel coronavirus SARS-CoV-2. Int J Antimicrob Agents 55:105923. https://doi.org/10.1016/j.ijantimicag.2020.105923.

24. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, Doudier B, Couzon J, Giordanengo V, Vieira VE, Dupont HT, Honore S, Colson P, Chabriere E, La Scola B, Rolain JM, Brouqui P, Raoult D. 20 March 2020. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. Int J Antimicrob Agents https://doi.org/10.1016/j.ijantimicag.2020.105949.

25. Lei J, Hilgenfeld R. 2017. RNA-virus proteases counteracting host innate immunity. FEMS Lett 591:3190–3210. https://doi.org/10.1016/1873-3468.12827.

26. Hung HC, Wang HC, Shih SR, Teng IF, Tseng CP, Hsu JT. 2011. Synergistic inhibition of enterovirus 71 replication by interferon and rupintrivir. J Infect Dis 203:1784–1790. https://doi.org/10.1093/infdis/jir174.

27. Tiew KC, He G, Aravapalli S, Mandadapu SR, Gunnam MR, Alliston KR, Lushington GH, Kim Y, Chang KO, Grouats WC. 2011. Design, synthesis, and evaluation of inhibitors of Norwalk virus 3C protease. Bioorg Med Chem Lett 21:5315–5319. https://doi.org/10.1016/j.bmcl.2011.07.016.

28. Mandadapu SR, Weerawarma PM, Gunnam MR, Alliston KR, Lushington GH, Kim Y, Chang KO, Grouats WC. 2012. Potent inhibition of norovirus 3C protease by peptidyl alpha-ketoamides and alpha-ketoheterocycles. Bioorg Med Chem Lett 22:4820–4826. https://doi.org/10.1016/j.bmcl.2012.05.055.

29. Kim Y, Liu H, Galasiti Kankanamalage AC, Weerasakara S, Hua DH, Grouats WC, Chang KO, Pedersen NC. 2016. Reversal of the progression of fatal coronavirus infection in cats by a broad-spectrum coronavirus protease inhibitor. PLoS Pathog 12:e1005531. https://doi.org/10.1371/journal.ppat.1005531.

30. Venkatachalam CM, Jiang X, Oldfield T, Waldman M. 2003. LigandFit: a novel method for the shape-directed rapid docking of ligands to protein active sites. J Mol Graph Model 21:289–307. https://doi.org/10.1016/s1093-3263(02)00164-x.

31. Kankanamalage ACG, Kim Y, Damalanka VC, Rathnayake AD, Fehr AR, Mezhabeen N, Battaile KP, Lovell S, Lushington GH, Perlman S, Chang KO, Grouats WC. 2018. Structure-guided design of potent and permeable inhibitors of MERS coronavirus 3CL protease that utilize a piperidine moiety as a novel design element. Eur J Med Chem 150:334–346. https://doi.org/10.1016/j.ejmech.2018.03.004.