Kinetic characterization and inhibitor screening for the proteases leading to identification of drugs against SARS-CoV-2

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ABSTRACT

The coronavirus (CoV) disease (COVID-19), caused by the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), has claimed many lives worldwide and is still spreading since December 2019. The 3C-like protease (3CL\textsuperscript{pro}) and papain-like protease (PL\textsuperscript{pro}) are essential for maturation of viral polyproteins in SARS-CoV-2 life cycle and thus regarded as key drug targets for the disease. In this study, 3CL\textsuperscript{pro} and PL\textsuperscript{pro} assay platforms were established and their substrate specificities were characterized. The assays were used to screen collections of 1068 and 2701 FDA-approved drugs. After excluding the externally used drugs which are too toxic, we totally identified 12 drugs as 3CL\textsuperscript{pro} inhibitors and 36 drugs as PL\textsuperscript{pro} inhibitors active at 10 μM. Among these inhibitors, 6 drugs were found suppressing SARS-CoV-2 with EC\textsubscript{50} below or close to 10 μM. This study enhances our understanding on the proteases and provides FDA approved drugs for prevention and/or treatment of COVID-19.

KEYWORDS: COVID-19, SARS-CoV-2, 3CL\textsuperscript{pro}, PL\textsuperscript{pro}, inhibitors, antivirals
The ongoing coronavirus (CoV) disease (COVID-19) has claimed many lives worldwide since the cases reported in December of 2019 (1, 2). The pathogen of COVID-19 is homologous to the severe acute respiratory syndrome (SARS)-associated CoV (SARS-CoV) that caused an outbreak in 2002–2003 (3, 4) and was thus named SARS-CoV-2 by WHO. In between, another outbreak of CoV disease that was started from the middle east countries in 2012 and spread to South Korea in 2015 was also caused by a human CoV named middle east respiratory syndrome CoV (MERS-CoV) (5, 6). The common syndrome of these CoV diseases is an atypical pneumonial infection featured by non-productive cough, high fever, and headache that may progress to generalized interstitial infiltrates in the lung. For drug repurposing against COVID-19, several approved antivirals which had been previously shown to inhibit SRAS-CoV and MERS-CoV were top choices of testing on SARS-CoV-2. For examples, Favipiravir, Ribavirin, and Penciclovir which are nucleotide analogues inhibiting viral RNA polymerases (7), as well as the human immunodeficiency virus-1 (HIV-1) protease inhibitors Lopinavir/Ritonavir which have been shown to inhibit SARS-CoV and MERS-CoV (8, 9) were earlier investigated but showed weak inhibition on the SARS-CoV-2 replication (10, 11). The drugs such as Chloroquine, an antimalarial drug that could block virus infection
by increasing endosomal pH required for membrane fusion between the virus and the host cell (12), Ivermectin, approved as an anti-parasitic agent (13) but also has antiviral activity against a broad range of viruses (14), and Arbidol, an entry inhibitor against influenza viruses and arboviruses (15), showed relatively lower EC$_{50}$ of 1.13, 2.5, and 4.11 $\mu$M, respectively, against SARS-CoV-2 in vitro (10, 16, 17). However, Chloroquine was shown to have no inhibitory effects on SARS-CoV-2 in human lung cells by in vitro assay (18). Although Hydroxychloroquine, a less toxic derivative of Chloroquine, was shown to inhibit SARS-CoV-2 in vitro (19), it showed no clinical benefit against COVID-19 (20).

Targeting enzymes essential for viral replication and life cycle of SARS-CoV-2 is a promising strategy. The main protease, also called 3C-like protease (3CL$^{\text{pro}}$), and the papain-like protease (PL$^{\text{pro}}$), which cleave 11 and 3 sites on the two polyproteins generated by SARS-CoV inside host cells to yield totally 16 mature non-structural proteins (NSPs), are essential for virus replication (21). To execute their functions, 3CL$^{\text{pro}}$ (NSP5) and PL$^{\text{pro}}$ (NSP3) first undergo autocleavage for maturation into active enzymes (22), so they have been used as targets for developing antivirals against SARS-CoV, MERS-CoV, and SARS-CoV-2 (23–25). The mature NSP12, a RNA-dependent RNA polymerase (RdRp), serves as the direct target of Remdesivir (26) that was previously shown to inhibit SARS-CoV and MERS-CoV (27).
Remdesivir antagonized SARS-CoV-2 viral replication with an EC$_{50}$ of 0.7 μM (10) and exhibited marginal (68%) benefit in clinical trials on COVID-19 patients (28).

Recently, a metallodrug ranitidine bismuth citrate was reported to suppress SARS-CoV-2 replication and relieve virus-associated pneumonia in Syrian hamsters by inhibiting the mature NSP13, a helicase (29).

3CL$^{\text{pro}}$ is chymotrypsin-like, but utilizes His-Cys dyad to cleave conserved sequences of (Leu, Met, Phe)-Gln↓(Ser, Ala, Gly) on the polyproteins of SARS-CoV (30). Several rationally designed peptidomimetics and FDA-approved or experimental drugs such as Disulfiram, Ebselen, Tideglusib, TDZD-8, Carmofur, and PX12 have been shown to inhibit 3CL$^{\text{pro}}$ and SARS-CoV-2 replication (31–34). Moreover, Boceprevir, GC-376, and Calpain inhibitors II, and XII have been demonstrated to inhibit SARS-CoV-2 viral replication by targeting 3CL$^{\text{pro}}$ (11). As reported recently, the cysteine protease inhibitors MDL-28170 (Calpain inhibitor III) and ONO 5334 (Cathepsin K inhibitor) were found to antagonize SARS-CoV-2 viral replication (35).

On the other hand, approved drugs as inhibitors of PL$^{\text{pro}}$, which can antagonize SARS-CoV-2 replication, have not been reported yet, although virtual screening studies have identified some candidate compounds (36). PL$^{\text{pro}}$ is a Cys protease with an Asp-His-Cys triad for cleaving sequences of Leu-(Asn, Lys, Arg)-Gly-Gly↓(Ala, Lys).

Recently, naphthalene-based inhibitors discovered previously (37) were shown to...
inhibit SARS-CoV-2 PL$_{pro}$ activity and SARS-CoV-2 replication (38). More recently, two peptidomimetic inhibitors with α,β-unsaturated ester warhead toward the active site Cys of SARS-CoV-2 PL$_{pro}$ were evaluated and their co-crystal structures with the proteases were solved to provide a framework for development of inhibitors with potential therapeutic value or drug repurposing (39).

To identify more marketed drugs with potential to treat COVID-19 patients by targeting 3CL$_{pro}$ and PL$_{pro}$, we established enzymatic assays for the proteases and characterized their kinetic constants and substrate specificities. We then performed a comprehensive screening on two large collections of drugs, one with 1068 and the other with 2701 FDA-approved drugs (the largest collection available so far). Using our 3CL$_{pro}$ and PL$_{pro}$ assays, we screened out 12 drugs as 3CL$_{pro}$ inhibitors and 36 drugs as PL$_{pro}$ inhibitors, respectively, which were active at 10 μM. By using plaque reduction assay, six drugs were shown to exhibit EC$_{50}$ below or close to 10 μM against the SARS-CoV-2 virus and their antiviral mechanisms were investigated as reported herein.

RESULTS

Purification and characterization of 3CL$_{pro}$ and PL$_{pro}$. 3CL$_{pro}$ was expressed as a thioredoxin fusion protein with a His-tag to facilitate its expression and
purification as shown by the reducing SDS-PAGE (Fig. 1A). Using the fluorogenic peptide Dabcyl-KTSAVLQSGFRKME-Edans containing a fluorescence quenching pair as the substrate, the $K_m$ value was determined to be 10.5±3.2 μM and the $k_{cat}$ value was 2.2±0.3 s$^{-1}$ (Fig. 1B). The $k_{cat}/K_m$ of SARS-CoV-2 3CL$^{pro}$ with this fluorogenic substrate was 0.21 μM$^{-1}$s$^{-1}$, 2-fold higher than that (0.11 μM$^{-1}$s$^{-1}$) for SARS-CoV 3CL$^{pro}$ using the same substrate (40).

PL$^{pro}$ was expressed with a N-terminal His-tag to facilitate its purification using NiNTA column as shown by the reducing SDS-PAGE (Fig. 1C). Using the commercially available fluorogenic peptide substrate Z-Arg-Leu-Arg-Gly-Gly-AMC, the $K_m$ value was measured to be 6.9±1.4 μM and the $k_{cat}$ value was 0.11±0.03 s$^{-1}$ (Fig. 1D). The catalytic efficiency $k_{cat}/K_m$ of 0.016 μM$^{-1}$s$^{-1}$ is 3-fold higher than 0.005 μM$^{-1}$s$^{-1}$ previously reported for SARS-CoV PL$^{pro}$ using the same substrate (41). The purified recombinant 3CL$^{pro}$ and PL$^{pro}$ were used to screen FDA-approved drugs to find their inhibitors as shown below.

Substrate specificities of 3CL$^{pro}$ and PL$^{pro}$ were then examined using the 12-aa peptides derived from their cleavage sequences on the polyproteins. The sequences of the 11 and 3 cleavage sites of the 3CL$^{pro}$ and PL$^{pro}$ are shown in Fig. 2A. When the 11 peptides were used as substrates, the rates of their cleavage by 3CL$^{pro}$ based on the HPLC assay to monitor the formation of two fragments from 100 μM of each peptide
and 0.75 μM 3CL\textsuperscript{pro} at pH 7.0 are displayed in Fig. 2B and summarized in Table 1. The fastest rate is 1.6 μM/sec, obtained from the peptide of N-terminal cleavage site. This is also the sequence used in the FRET peptide substrate for assaying the 3CL\textsuperscript{pro} kinetics. The cleavage rates using 100 μM of 3 peptides as substrates of 0.75 μM PL\textsuperscript{pro} are shown in Fig. 2C and summarized in Table 1. PL\textsuperscript{pro} cleaved its own N-terminal cleavage site most efficiently at a rate of 0.09 μM/sec, its own C-terminal site at 0.01 μM/sec, while that of the site between NSP1 and 2 at 0.003 μM/sec, the slowest.

Screening of 1068 and 2701 FDA-approved drugs to find inhibitors of SARS-CoV-2 3CL\textsuperscript{pro}. After screening the 1068 FDA-approved drugs by using the FRET assay, the compounds that inhibited 3CL\textsuperscript{pro} are colored in yellow and that inhibited both 3CL\textsuperscript{pro} and PL\textsuperscript{pro} are colored in green (please see Dataset S1). After excluding the highly toxic externally used drugs, we found 1 drug, Tolcapone, that could inhibit 3CL\textsuperscript{pro} with an IC\textsubscript{50} of 7.9 μM (Table S1 and Fig. 3A). However, Tolcapone only showed a weak antiviral activity at 50 μM (Figure not shown).

The 2701 FDA-approved drugs were screened against 3CL\textsuperscript{pro} and the compounds that inhibited 3CL\textsuperscript{pro} are colored in yellow and that inhibited both 3CL\textsuperscript{pro} and PL\textsuperscript{pro} are colored in green (please see Dataset S2). After excluding the externally used medicines, 11 hits (Table S2) were identified active in inhibiting 3CL\textsuperscript{pro} at 10 μM.
Among these, only Levothyroxine and Manidipine-2HCl in addition to the previously reported 3CL\textsuperscript{pro} inhibitors were shown to inhibit virus replication by plaque reduction assay (see below). Here, the dose-dependent 3CL\textsuperscript{pro}-inhibition curves were used to determine the IC\textsubscript{50} values of Levothyroxine and Manidipine-2HCl being 19.2±1.2 and 10.4±1.6 μM, respectively (Fig. 3B and 3C).

Screening on 1068 and 2701 FDA-approved drugs to find inhibitors of SARS-CoV-2 PL\textsuperscript{pro}. Through screening the 1068 FDA-approved drugs, we found 10 hits (Table S3) that could inhibit PL\textsuperscript{pro} activity when 10 μM was used in the assay. All the drugs screened are listed in Dataset S1 and the compounds that inhibited PL\textsuperscript{pro} are colored in blue and that inhibited both 3CL\textsuperscript{pro} and PL\textsuperscript{pro} are colored in green. However, only two drugs, Maprotiline and Reserpine, showed antiviral activity in the plaque reduction assay as shown later. The dose-dependent inhibition curves of Maprotiline and Reserpine were determined as shown in Fig. 4A and 4B, and the derived IC\textsubscript{50} values were 9.7±0.3 and 5.7±0.7 μM, respectively.

Then 2701 FDA-approved drugs were screened against PL\textsuperscript{pro} and 26 additional hits (Table S4) were identified. All the 2701 drugs screened are listed in Dataset S2 and the compounds that inhibited PL\textsuperscript{pro} are colored in blue and that inhibited both 3CL\textsuperscript{pro} and PL\textsuperscript{pro} are colored in green. Among these 26 hits, Levothyroxine, Loperamide, Manidipine-2HCl, and Proanthocyanidin were shown to inhibit virus
replication by the plaque reduction assay as shown later. The dose-dependent
inhibition curves of these 4 drugs were determined (Fig. 4C–F). Based on the data, the
IC_{50} values for Levothyroxine, Loperamide, Manidipine-2HCl, and Proanthocyanidin
were 15.3±4.2, 33.5±5.8, 14.2±1.7, and 2.4±0.3 μM, respectively.

Antiviral EC_{50} measurements of the inhibitors. As mentioned above, a total of
46 drugs from the two drug collections were identified as either 3CL^{pro} or PL^{pro}
inhibitors and 2 drugs inhibited both at the concentration of 10 μM. The antiviral
activities of these 46 drugs (10 μM) were tested using plaque reduction assay and the
active compounds are colored red in Supplementary Tables. Initially, to maximize the
chances to identify antivirals, we pre-treated the cells with each drug, added each drug
during the virus infection and after virus removal. By this full-time treatment as
shown in Fig. 5A, we identified two 3CL^{pro}-inhibiting drugs, Levothyroxine and
Manidipine-2HCl, as well as six PL^{pro}-inhibiting drugs, Levothyroxine, Loperamide,
Manidipine-2HCl, Maprotiline, Reserpine, and Proanthocyanidin, effective in
reducing viral plaques. Next, two other treatment methods were performed to
distinguish the stages for the drugs against SARS-CoV-2. As shown in Fig. 5A,
“entry” was to pre-treat the cells and virus during infection with each drug but without
adding the drug after removal of virus, and the “post-entry” means each drug was
added to the cells only after virus infection. By using these treatment methods, we
suspected Loperamide, Manidipine-2HCl, and Maprotiline antagonized viral replication by mainly inhibiting the viral protease(s) because post-entry treatment could still inhibit the virus. On the contrast, Levothyroxine and Proanthocyanidin suppressed the viral plaques probably by blocking the virus entry because post-entry treatment could not inhibit the virus. Therefore, the measurements of EC_{50} were performed with “post-entry” treatment for Loperamide, Manidipine-2HCl, and Maprotiline and “entry” treatment for Levothyroxine and Proanthocyanidin as shown in Fig. 5A. For Reserpine, apparent antiviral activity was observed only when full-time treatment was applied and the EC_{50} was measured using full-time treatment (entry plus post-entry). The EC_{50} values for these drugs were determined accordingly using plaque reduction assay (Fig. S1), and the plots of concentration-dependent virus plaque reduction under serially diluted drug concentrations are shown in Fig. 5B–G. The derived EC_{50} values for Loperamide, Manidipine-2HCl, Maprotiline, Levothyroxine, Proanthocyanidin, and Reserpine, were 11.4±1.6, 14.5±0.8, 9.3±0.1, 7.0±2.2, 2.5±0.4, and 6.6±1.5 μM, respectively. The CC_{50} values for Loperamide and Maprotiline were 56.4±0.7 and 31.8±0.4 μM, respectively, and those for other drugs were all >100 μM. The derived SI for Reserpine (SI>15.2), Levothyroxine (SI>14.3), and Proanthocyanidin (SI>40) were higher than 10, while those for Loperamide (SI=4.9), Manidipine-2HCl (SI>6.9), and Maprotiline (SI=3.4) were below 10.
DISCUSSION

As demonstrated here, we have purified the recombinant 3CL$^{pro}$ and PL$^{pro}$ and monitored their activities using the fluorogenic peptide substrates (Fig. 1A–D). The kinetic efficiencies ($k_{\text{cat}}/K_m$) of two SARS-CoV-2 proteases are higher than that of SARS-CoV proteases. The cleavage rates of the examined peptides derived from their respective cleavage-site sequences reveal that the N-terminal auto-cleavage reactions are the fastest for both 3CL$^{pro}$ and PL$^{pro}$ (Fig. 2A–C). This is probably due to the fact that the maturation of NSPs in two polyproteins requires first cleavage of the proteases’ N-termini. After cleavage of their N-termini, the proteases become more flexible to undergo C-terminal cleavage toward fully active 3CL$^{pro}$ and PL$^{pro}$ which subsequently cleave other sites on the polyproteins. Our data also reveal the most preferred recognition sequences of Leu(P2)-Gln(P1)$\downarrow$Ser(P1′) and Lys(P3)-Gly(P2)-Gly(P1)$\downarrow$Ala(P1′) for SARS-CoV-2 3CL$^{pro}$ and PL$^{pro}$, respectively.

Using these assay platforms, we have screened the collections of 1068 and 2701 FDA-approved drugs and found 1 hits from 1068 drugs and additional 11 hits from 2701 drugs, which were active at 10 μM against 3CL$^{pro}$. Among the hits, 3 drugs, Disulfiram, Ebselen, and Boceprevir, have been previously reported (11, 33) and 1 drug Tolcapone was previously found active in inhibiting feline CoV (42). Moreover, 10 hits from 1068 drugs and additional 26 hits from 2701 drugs, which displayed
PL<sub>pro</sub>-inhibiting activity at 10 μM were obtained from the screening. The inhibition concentration-dependent curves against the 3CL<sub>pro</sub> (Fig. 3) and PL<sub>pro</sub> (Fig. 4) for those drugs showing antiviral activities were determined. To be noted, although Levothyroxine and Manidipine-2HCl inhibit both 3CL<sub>pro</sub> and PL<sub>pro</sub> in the in-vitro assay, their antiviral mechanisms vary. For example, Levothyroxine exerts its antiviral activities at the entry step (EC<sub>50</sub>=7.0±2.2 μM) (Fig. 5E) instead of the post-entry stage (EC<sub>50</sub>&gt;20 μM), while Manidipine-2HCl only exerts its antiviral activities at the post-entry stage (EC<sub>50</sub>=14.5±0.8 μM) (Fig. 5C). Interestingly, among the three PL<sub>pro</sub> inhibitors showing anti-SARS-CoV-2 activities, Proanthocyanidin, like Levothyroxine, also showed strong antiviral activities at the entry step with the EC<sub>50</sub> values of 2.5±0.4 μM (Fig. 5F). Therefore, Levothyroxine and Proanthocyanidin might block the entry of virus, which requires further experiments to prove. Another 2 PL<sub>pro</sub>-only inhibitors, Loperamide and Maprotiline, showed antiviral activities at the post-entry stage with the EC<sub>50</sub> values of 11.4±1.6 and 9.3±0.1 μM, respectively (Fig. 5B and 5D), but not the entry step, implicating that they certainly inhibit virus replication inside the host cells. On the contrast, Reserpine exerts its antiviral activity only when it was treated full-time (entry plus post-entry), suggesting that both of its PL<sub>pro</sub>-inhibiting and virus entry-inhibiting activities might contribute to its anti-virus effect (EC<sub>50</sub>=6.6±1.5 μM) (Fig. 5G). Therefore, the use of full-time, entry, and
post-entry treatments allows determination of the stages where the antiviral drugs exert their inhibitory effects.

Loperamide, sold under the brand name of Imodium, is a medication used to decrease the frequency of diarrhea. It is an opioid-receptor agonist and acts on the \( \mu \)-opioid receptors in the myenteric plexus of the large intestine to decrease the tone of the longitudinal and circular smooth muscles of the intestinal wall (43), thereby allowing more water to be absorbed from the fecal matter. Manidipine-2HCl is a calcium channel blocker (dihydropyridine type) that is used clinically as an antihypertensive (44). Maprotiline, sold under the brand name Ludiomil, is a tetracyclic antidepressant that is used in the treatment of depression. It is known to function as a norepinephrine reuptake inhibitor and also a strong antagonist of the H1 receptor (45). Reserpine is one of dozens of indole alkaloids isolated from the plant Rauwolfia serpentine. It is used for the treatment of high blood pressure by irreversibly blocking the H\(^+\)-coupled vesicular monoamine transporters, VMAT1 and VMAT2. It is the blockade of neuronal VMAT2 by reserpine that inhibits uptake and reduces stores of the monoamine neurotransmitters norepinephrine, dopamine, serotonin and histamine in the synaptic vesicles of neurons (46). Reserpine was identified by our previous study (47) to inhibit SARS-CoV replication, but the target was not known until now to be PL\text{pro} and virus entry. Its EC\(_{50}\) values against
SARS-CoV was measured to be 3.4 μM (47). Six natural derivatives of Reserpine were previously shown to exhibit activities toward SARS-CoV at less than 100 μM (47), and their activities against SARS-CoV-2 could be further examined in the future.

Levothyroxine, also known as L-thyroxine, is a manufactured form of the thyroid hormone thyroxine (T4), used to treat thyroid hormone deficiency, including the severe form known as myxedema coma (48). Proanthocyanidins, a class of polyphenols found in a variety of plants, can function as antioxidants to remove harmful free oxygen radicals from cells. Therefore, Proanthocyanidins have been known to confer significant health benefits as reported by several studies using human and animal models (49).

In summary, by using the 3CL\(^{\text{pro}}\) and PL\(^{\text{pro}}\) fluorescence assays, we identify 6 FDA-approved drugs which could suppress SARS-CoV-2. Considering the frequent uptake of Loperamide and Proanthocyanidins in some populations, it will be interesting to know the effectiveness of these drugs in prophylactic prevention of SARS-CoV-2 infection. We thus provide here candidates for prevention and/or treatment of COVID-19.

**MATERIALS AND METHODS**

**Materials.** Fluorogenic peptide substrate Dabcyl-KTSAVLQSGFRKME-Edans of 3CL\(^{\text{pro}}\) was synthesized by Yuan Yu Ltd. (Taiwan). The fluorogenic substrate
peptide (z-Arg-Leu-Arg-Gly-Gly-AMC) for PL\textsuperscript{pro} was purchased from BACHEM (Bubendorf, Switzerland). The collections of 1068 and 2701 FDA-approved drugs were purchased from Target Molecule Corp. (Boston, USA) and Selleck Chemicals (Houston, USA), respectively. After screening, the active compounds in bulk size (e.g. 25 mg) were purchased from the companies for IC\textsubscript{50}, EC\textsubscript{50}, and CC\textsubscript{50} measurements. The plasmid mini-prep kit, DNA gel extraction kit, and Ni-NTA resin were purchased from Qiagen (Hilden, Germany). FXa and the protein expression kit including pET32a and pET16b vectors as well as competent JM109 and BL21(DE3) cells were obtained from Novagen (Merck KGaA, Darmstadt, Germany). The SARS-CoV-2 virus used in this study was SARS-CoV-2/NTU13/TWN/human/2020 (Accession ID EPI_ISL_422415) as described (50). All commercial buffers and reagents were of the highest grade.

**Expression and purification of SARS-CoV-2 3CL\textsuperscript{pro}**. The gene encoding SARS-CoV-2 3CL\textsuperscript{pro} was synthesized by Genewiz Co. (South Plainfield, NJ, USA) and cloned into the pET32a vector by using polymerase chain reaction (PCR) with the forward primer 5'-CAT GCC ATG GCT ATC GAG GGA AGG AGT GGT TTT AGG AAA ATG GC-3' containing FXa and NcoI cleavage sequences and the reverse primer 5'-CCG CTC GAG TTA TTG GAA GGT AAC ACC AGA G-3' containing XhoI site sequence (the underlined sequences encode the N- and C-terminus of 3CL\textsuperscript{pro},...
respectively). The vector pET32a also encodes thioredoxin and His-tag at the N-terminus of the target protein. The recombinant 3CL\textsuperscript{pro} plasmid was then used to transform the \textit{E. coli} JM109 competent cells and the transformed cells were streaked on a Luria-Bertani (LB) agar plate containing 100 μg/mL ampicillin. Ampicillin-resistant colonies were selected from the agar plate and sequenced to detect the 3CL\textsuperscript{pro} gene. The correct construct was subsequently transformed to \textit{E. coli} BL21(DE3) for protein expression. The 5-mL overnight culture of a single transformant was used to inoculate 500 mL of fresh LB medium containing 100 μg/mL ampicillin. The cells were grown to OD\textsubscript{600} = 0.6 and induced with 1 mM isopropyl-β-thiogalactopyranoside. After 4–5 h, the cells were harvested by centrifugation at 7,000 g for 15 min. The 3CL\textsuperscript{pro} purification was conducted at 4 °C. The cell paste obtained from 1 L cell culture was suspended in 40 mL lysis buffer containing 25 mM Tris-HCl, pH 7.5, and 150 mM NaCl. French-press instrument (AIM-AMINCO spectronic Instruments, Cambridge scientific products, MA, USA) was used to disrupt the cells at 12,000 psi. The lysis solution was centrifuged and the debris was discarded. The cell-free extract was loaded onto a 10-mL Ni-NTA column equilibrated with the same buffer containing 5 mM imidazole. The column was washed with 5 mM imidazole followed by 30 mM imidazole-containing buffer. The His-tagged 3CL\textsuperscript{pro} was eluted with the
buffer containing 300 mM imidazole. The protein solution was dialyzed against 1 L lysis buffer two times. After overnight dialysis, the tagged 3CL<sup>pro</sup> was treated with FXa protease to remove thioredoxin and His-tag and the mixture was loaded onto a Ni-NTA column equilibrated with the buffer containing 5 mM imidazole. The tag-free 3CL<sup>pro</sup> in flow through was dialyzed against the buffer without imidazole (12 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1 mM EDTA, and 2 mM DTT) and stored at -70 °C. The enzyme concentrations used in experiments were determined based on the absorbance at 280 nm.

**Expression and purification of SARS-CoV-2 PL<sup>pro</sup>.** The gene encoding SARS-CoV-2 PL<sup>pro</sup> was synthesized by Genewiz Co. (South Plainfield, NJ, USA) and cloned into the pET16b vector by using polymerase chain reaction (PCR) with the forward primer 5’-CAT GCC ATG GCC CAT CAT CAT CAT CAT CAT CAT GAA GTG AGG ACT ATT AAG GTG-3’ containing NcoI cleavage sequence and reverse primer 5’-CCG CTC GAG TTA TAT GGT TGT TGT GTA ACT GTT TTC TTT GTA G-3’ containing XhoI site sequence (the underlined sequences encode the N- and C-terminus of PL<sup>pro</sup>, respectively). The forward primer also encodes extra MAHHHHHHH at the N-terminus of target protein. The recombinant PL<sup>pro</sup> plasmid was then used to transform *E. coli* JM109 competent cells and the transformed cells were streaked on a Luria-Bertani (LB) agar plate containing 100 μg/mL ampicillin.
Ampicillin-resistant colonies were selected from the agar plate and sequenced for the

$PL^{pro}$ gene. The correct construct was subsequently transformed to *E. coli* BL21(DE3)

for protein expression. The 5-mL overnight culture of a single transformant was used
to inoculate 500 mL of fresh LB medium containing 100 μg/mL ampicillin. The cells
were grown to OD$_{600}$ = 0.6 and induced with 1 mM

isopropyl-β-thiogalactopyranoside. After 4–5 h, the cells were harvested by

centrifugation at 7,000 g for 15 min.

The $PL^{pro}$ purification was conducted at 4 °C. The cell paste obtained from 1 L
cell culture was suspended in 40 mL lysis buffer containing 25 mM Tris-HCl, pH 7.5
and 150 mM NaCl. French-press instrument (AIM-AMINCO spectronic Instruments,
Cambridge scientific products, MA, USA) was used to disrupt the cells at 12,000 psi.
The lysis solution was centrifuged and the debris was discarded. The cell free extract
was loaded onto a 10 mL Ni-NTA column equilibrated with the same buffer
containing 5 mM imidazole. The column was washed with 5 mM imidazole followed
by 30 mM imidazole-containing buffer. The His-tagged $PL^{pro}$ was eluted with the
buffer containing 300 mM imidazole. The His-tagged $PL^{pro}$ in flow through was
dialedyzed into a buffer containing 12 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1 mM
EDTA, and 2 mM DTT for storage at -70 °C. The enzyme concentrations used in all
experiments were determined from the absorbance at 280 nm.
Kinetics of 3CL<sup>pro</sup> and PL<sup>pro</sup> measured using the fluorogenic substrates. The kinetic measurements were performed in the buffer (20 mM Bis-Tris, pH 7.0) at 25 °C. Enhanced fluorescence due to cleavage of the fluorogenic substrate peptide (Dabcyl-KTSAVLQSGFRKME-Edans) for 3CL<sup>pro</sup> or (z-Arg-Leu-Arg-Gly-Gly-AMC) for PL<sup>pro</sup> was monitored at 538 and 460 nm, respectively, with excitation at 355 nm using a fluorescence plate reader (Fluoroskan Ascent, ThermoLabsystems, Sweden). The enzyme concentration used for measuring K<sub>m</sub> and V<sub>max</sub> values was 35 nM for 3CL<sup>pro</sup> and 75 nM for PL<sup>pro</sup> and the substrate concentrations were from 0.5–5-fold K<sub>m</sub> values. 3CL<sup>pro</sup> substrate concentration was determined by using the extinction coefficients 5438 M<sup>-1</sup> cm<sup>-1</sup> at 336 nm (Edans) and 15,100 M<sup>-1</sup> cm<sup>-1</sup> at 472 nm (Dabcyl). PL<sup>pro</sup> substrate concentration was determined by using the extinction coefficient of 17800 M<sup>-1</sup> cm<sup>-1</sup> at 354 nm (AMC). The initial rates within 10% substrate consumption under different substrate concentrations were used to calculate the kinetic parameters by fitting with Michaelis-Menten equation (V=V<sub>max</sub>[S]/K<sub>m</sub>+[S]) using KaleidaGraph computer program. For each data point, the measurements were repeated three times to yield the averaged number and the standard deviation.

Peptide substrate specificity measurements. Peptides for testing as substrates were chemically synthesized by Mission Biotech (Taipei, Taiwan) with a solid-phase methodology based on the sequences of cleavage sites on viral polyproteins (Fig. 2A).
Reactions (100 μL) contained 0.75 μM protease and 100 μM of each peptide in the buffer of 20 mM Bis-Tris (pH 7.0) for 3CL\textsuperscript{pro} or the buffer of 20 mM HEPES (pH 7.0) for PL\textsuperscript{pro}. Following incubation at 37°C for 0, 2.5, 5, and 10 min, reactions were stopped by adding 5 μl trifluoroacetic acid and applied to a C18 reverse-phase HPLC column (250 x 4.6 mm; Hichrom, Berkshire, UK). The column was eluted at room temperature with a linear acetonitrile gradient in the presence of 0.1% (v/v) trifluoroacetic acid at a flow rate of 1.0 ml/min. The areas of the product peaks monitored at 214 nm were integrated to calculate the cleavage rate of each peptide substrate under the catalysis of 3CL\textsuperscript{pro} or PL\textsuperscript{pro}.

**Inhibition assay.** The collections of drugs at 10 μM were screened for inhibiting the proteases and the active inhibitors were confirmed three times. Only the IC\textsubscript{50} values of the inhibitors which showed antiviral activity were measured in reaction mixtures containing 2.5 nM 3CL\textsuperscript{pro} with 6 μM fluorogenic substrate in a buffer of 20 mM Bis-Tris (pH 7.0) or 75 nM PL\textsuperscript{pro} with 10 μM fluorogenic substrate in a buffer of 20 mM Bis-Tris (pH 7.5) in the absence and presence of various concentrations of the inhibitors. The fluorescence changes resulted from the reactions were followed with time at 538 nm with excitation at 355 nm for 3CL\textsuperscript{pro} or at 460 nm with excitation at 355 nm for PL\textsuperscript{pro} using the fluorescence plate reader. The initial velocities of the inhibited reactions were plotted against the different inhibitor concentrations to yield
the IC₅₀ value by fitting with the equation: \( A(I) = A(0) \times \left\{ 1 - \frac{I}{I + IC_{50}} \right\} \). In this equation, \( A(I) \) is the enzyme activity with inhibitor concentration \( I \), \( A(0) \) is the enzyme activity without inhibitor, and \( I \) is the inhibitor concentration. For each data point, the measurements were repeated three times to yield the averaged number and the standard deviation.

**Cell-based assay.** For the SARS-CoV-2 plaque reduction assay, Vero E6 cells were seeded to a 24-well culture plate in DMEM with 10% FBS and antibiotics one day before infection. VeroE6 cells were infected by SARS-CoV-2 virus (50–100 plaque forming unit, pfu) for 1 h at 37°C. After removal of virus inoculum, the cells were washed once with PBS and overlaid with 1 mL overlay medium containing 1% methylcellulose for 5 days at 37°C. After 5 days, the cells were fixed with 10% formalin overnight. After removal of overlay media, the cells were stained with 0.5% crystal violet and the plaques were counted. The percentage of inhibition was calculated as \( 1 - \frac{V_D}{V_C} \) x 100%, where \( V_D \) and \( V_C \) refer to the virus titer in the presence and absence of the inhibitors, respectively. The minimal concentrations of compounds required to reduce 50% of plaque numbers (EC₅₀) were calculated by regression analysis of the dose-response curves generated from plaque assays. For each data point, the measurements were repeated three times to yield the averaged number and the standard deviation. To determine the stages where the drugs exert its...
antiviral effects, the drugs can be added before infection, during infection, or after infection as shown in Fig. 5A.

Cytotoxicity of the inhibitors was determined by using the acid phosphatase (ACP) assay. Briefly, Vero E6 cells were seeded onto a 96-well culture plate at a concentration of $2 \times 10^4$ cells per well. Next day, medium was removed and each well was washed once with PBS before adding DMEM containing 2% FBS and different concentrations of inhibitors. Next, DMEM containing 2 μg/mL TPCK-trypsin was added. After 1 hr of incubation at 37°C, medium was removed and cells were washed by PBS. Then, DMEM containing 2% FBS and different concentrations of each compound was added. After 3 days of incubation at 37°C, medium was removed and each well was washed once with PBS. Next, buffer containing 0.1 M sodium acetate (pH = 5.0), 0.1% Triton X-100, and 5 mM p-nitrophenyl phosphate was added. After incubating at 37 °C for 2 hr, 1 N NaOH was added to stop the reaction. The absorbance was then determined by ELISA reader (VERSAmax, Molecular Devices, Sunnyvale, CA) at a wavelength of 405 nm. The percentage of cytotoxicity was calculated using the following formula: 

$$\text{cytotoxicity} \% = \left[1 - \left(\frac{A_t}{A_s}\right)\right] \times 100\%,$$ 

where $A_t$ and $A_s$ refer to the absorbance of a tested substance and solvent control, respectively. The 50% cytotoxicity concentration (CC$_{50}$) was defined as the concentration reducing
50% of cell viability. For each data point, the measurements were repeated three times to yield the averaged number and the standard deviation.

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We declare that we have no conflicts of interest.

C.J.K. and P.H.L. designed the research; C.J.K., T.L.C. H.C.K., Y.M.T., Y.K.L., and M.C.H. performed the research; C.J.K. and S.Y.C. analyzed the data; L.W. provided the drug libraries; and P.H.L. wrote the paper.
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Table 1: Cleavage rates of the peptides by SARS-CoV-2 3CL\textsuperscript{pro} and PL\textsuperscript{pro}.

| Protease          | Peptide             | Cleavage rate (\(\mu\)M/sec) |
|-------------------|---------------------|-------------------------------|
| SARS-CoV-2 3CL\textsuperscript{pro} | TSAVLQSGFRKM         | 1.60\(\pm\)0.12              |
|                   | SGVTFSQAVKRT         | 0.12\(\pm\)0.03              |
|                   | KVATVQSKMSDV         | 0.03\(\pm\)0.005             |
|                   | NRATLQAIASEF         | 0.07\(\pm\)0.02              |
|                   | SAVKLQNNELSP         | 0.02\(\pm\)0.003             |
|                   | ATVR1QLAGNATE        | 0.15\(\pm\)0.011             |
|                   | REPMLQSADAQS         | 0.03\(\pm\)0.01              |
|                   | PHTVLQAVGACV         | 0.05\(\pm\)0.012             |
|                   | NVATLQAENVTVG        | 0.01\(\pm\)0.005             |
|                   | TFTRLQSLENVVA        | 0.11\(\pm\)0.015             |
|                   | FYPKLQSSQAWQ         | 0.26\(\pm\)0.02              |
| SARS-CoV-2 PL\textsuperscript{pro} | RELNGGAYTRYV         | 0.003\(\pm\)0.001            |
|                   | FTLKGGAPTKVT         | 0.09\(\pm\)0.012             |
|                   | IALKGGKIVNNW         | 0.01\(\pm\)0.004             |
FIGURE LEGENDS

FIG 1 Preparation and characterization of SARS-CoV-2 3CL\textsuperscript{pro} and PL\textsuperscript{pro}. (A) SDS-PAGE analysis for the purification of the recombinant 3CL\textsuperscript{pro}. Lane M represents the molecular mass markers of 100, 75, 63, 48, 35, 28, and 17 kDa. Lanes 1 and 2 show the cell lysate without and with IPTG induction to overexpress 3CL\textsuperscript{pro} fused with thioredoxin and His-tag, respectively. Lane 3 is the tagged protease after Ni-NTA column chromatography. Lane 4 represents the tagged 3CL\textsuperscript{pro} treated with FXa to remove thioredoxin and His-tag. Two extra bands, intact 3CL\textsuperscript{pro} and the thioredoxin-His-tag with lower molecular mass, appear in this lane. Lane 5 shows the purified tag-free 3CL\textsuperscript{pro} after using Ni-NTA column. (B) Kinetic constants of 3CL\textsuperscript{pro}. The reaction initial rates of the protease were plotted against different substrate concentrations to yield the V\textsubscript{max} and K\textsubscript{m} values. (C) SDS-PAGE analysis for purifying the recombinant PL\textsuperscript{pro}. Lane M represents the molecular mass markers of 100, 75, 63, 48, 35, 28, and 17 kDa. Lanes 1 and 2 show the cell lysates without and with IPTG induction to overexpress PL\textsuperscript{pro} fused with thioredoxin and His-tag, respectively. Lane 3 is the tagged PL\textsuperscript{pro} after Ni-NTA column chromatography. (D) Kinetic constants of PL\textsuperscript{pro}. The reaction initial rates of PL\textsuperscript{pro} were plotted against different substrate concentrations.
concentrations to yield the \( V_{\text{max}} \) and \( K_{\text{m}} \) values.

**FIG 2** Substrate specificities of SARS CoV-2 3CL\(^{\text{pro}}\) and PL\(^{\text{pro}}\). (A) On the PPIab containing 16 non-structural proteins (NSPs), the boxed sequences in grey and black represent the cleavage sites of PL\(^{\text{pro}}\) and 3CL\(^{\text{pro}}\), respectively. Each synthetic peptide harbors 12 residues of each cleavage site. Peptide cleavage rates by 3CL\(^{\text{pro}}\) and PL\(^{\text{pro}}\) are shown in (B) and (C), respectively.

**FIG 3** Screening and IC\(_{50}\) measurements of the 3CL\(^{\text{pro}}\) inhibitors. (A–C) The IC\(_{50}\) values of FDA-approved drugs, Tolcapone, Levothyroxine, and Manidipine-2HCl, were measured to be 7.9±0.9, 19.2±1.2, and 10.4±1.6, respectively, based on the inhibitor concentration-dependent curves.

**FIG 4** Screening and IC\(_{50}\) measurements of the PL\(^{\text{pro}}\) inhibitors. (A–D) FDA-approved drugs, Maprotiline, Reserpine, Levothyroxine, Loperamide, Manidipine-2HCl, and Proanthocyanidin were screened out to be the PL\(^{\text{pro}}\) inhibitors with IC\(_{50}\) of 9.7±0.3, 5.7±0.7, 15.3±4.2, 33.5±5.8, 14.2±1.7, and 2.4±0.3 \( \mu \)M, respectively, based on the inhibitor concentration-dependent curves.
FIG 5 EC₅₀ measurements of 3CLₚₚ and PLₚₚ inhibitors based on the plaque reduction assay. (A) Schematic illustration of the full-time, entry, and post-entry experiments delineates the stage where the test drugs were added. (B–G) Six drugs identified from 1,068 and 2,701 drug collections showed apparent antiviral activity against the SARS-CoV-2 virus with EC₅₀ of 11.4±1.6, 14.5±0.8, 9.3±0.1, 7.0±2.2, 2.5±0.4, and 6.6±1.5 μM for (B) Loperamide, (C) Manidipine-2HCl, (D) Maprotiline, (E) Levothyroxine, (F) Proanthocyanidin, and (G) Reserpine, respectively, as calculated from the dose-dependent plaque reduction percentages (Fig. S1), represented by filled black circles. The sigmoidal fitting curves are shown with solid lines. The CC₅₀ values for (B) Loperamide and (D) Maprotiline were 56.4±0.7 and 31.8±0.4 μM, respectively, while those for other drugs were >100 μM, based on the cell viability inhibition percentages, represented by open circles. The fitting curves are shown with dotted lines.
Figure 1
Figure 2

A. Tolcapone_3CLpro
IC$_{50}$ = 7.9±0.9 μM

B. Levothyroxine_3CLpro
IC$_{50}$ = 19.2±1.2 μM

Figure 3

C. Manidipine_3CLpro
IC$_{50}$ = 10.4±1.6 μM
A. Maprotiline$_{PL^{pro}}$
   IC$_{50}$ = 9.7±0.3 μM

B. Reserpine$_{PL^{pro}}$
   IC$_{50}$ = 5.7±0.7 μM

C. Levothyroxine$_{PL^{pro}}$
   IC$_{50}$ = 15.3±4.2 μM

D. Loperamide$_{PL^{pro}}$
   IC$_{50}$ = 33.5±5.8 μM

E. Manidipine$_{PL^{pro}}$
   IC$_{50}$ = 14.2±1.7 μM

F. Proanthocyanidin$_{PL^{pro}}$
   IC$_{50}$ = 2.4±0.3 μM
Figure 4

A. Pretreat cell | Add Virus | 1X PBS wash

| 37°C/1hr | 37°C/1hr | 37°C/24hr |
|----------|----------|-----------|
| Reserpine | Full-time | Entry |
| Loperamide/Proanthocyanidin | Post-entry |

B. Loperamide (μM)

C. Mapiroline (μM)

D. Proanthocyanidin (μM)

E. Levothyroxine (μM)

F.曼地昔 (μM)

G. Reserpine (μM)
