Evaluation of SARS-CoV-2 Main Protease Inhibitors Using a Novel Cell-Based Assay

Wenyue Cao, Chia-Chuan Dean Cho, Zhi Zachary Geng, Namir Shaabani, Xinyu R. Ma, Erol C. Vatansever, Yugendar R. Alugubelli, Yuying Ma, Sankar P. Chaki, William H. Ellenburg, Kai S. Yang, Yuchen Qiao, Robert Allen, Benjamin W. Neuman, Henry Ji, Shiqing Xu, and Wenshe Ray Liu

Cite This: ACS Cent. Sci. 2022, 8, 192–204

ABSTRACT: As an essential enzyme of SARS-CoV-2, main protease (M^{Pro}) triggers acute toxicity to its human cell host, an effect that can be alleviated by an M^{Pro} inhibitor. Using this toxicity alleviation, we developed an effective method that allows a bulk analysis of the cellular potency of M^{Pro} inhibitors. This novel assay is advantageous over an antiviral assay in providing precise cellular M^{Pro} inhibition information to assess an M^{Pro} inhibitor. We used this assay to analyze 30 known M^{Pro} inhibitors. Contrary to their strong antiviral effects and up to 10 μM, 11a, calpain inhibitor II, calpain XII, ebselen, bepridil, chloroquine, and hydroxychloroquine showed relatively weak to undetectable cellular M^{Pro} inhibition potency implicating their roles in interfering with key steps other than just the M^{Pro} catalysis in the SARS-CoV-2 life cycle. Our results also revealed that MPI5, MPI6, MPI7, and MPI8 have high cellular and antiviral potency. As the one with the highest cellular and antiviral potency among all tested compounds, MPI8 has a remarkable cellular M^{Pro} inhibition IC_{50} value of 31 nM that matches closely to its strong antiviral effect with an EC_{50} value of 30 nM. Therefore, we cautiously suggest exploring MPI8 further for COVID-19 preclinical tests.

INTRODUCTION

COVID-19 has paralyzed much of the world. As of December 9, 2021, the total confirmed infections have reached above 267 million, and the total death toll has exceeded 5.2 million worldwide. With vaccines available for COVID-19, many countries have been conducting immunization campaigns hoping that herd immunity will be achieved when the majority of the population is vaccinated. Current COVID-19 vaccines are targeting the Spike protein of SARS-CoV-2, the pathogen of COVID-19. Spike is a weakly conserved protein in a highly mutable RNA virus. Although SARS-CoV-2 shares overall 82% genome sequence identity with SARS-CoV, Spike has only 76% protein sequence identity shared between two origins. The highly mutable nature of Spike has also been corroborated by the continuous identification of new SARS-CoV-2 variants with Spike mutations. The most notable are Alpha, Beta, Delta, and Omicron variants. Accumulated evidence has shown an attenuated activity of approved vaccines against some new SARS-CoV-2 variants. Booster vaccines might be developed for new virus variants. However, the situation will likely turn into an incessant race between viral mutation and vaccine development. The focus on vaccine development that is preventative toward COVID-19 has largely obscured the development of targeted therapeutics that are needed for treating patients with severe symptoms. By targeting a conserved gene in SARS-CoV-2, a small-molecule antiviral will likely be more successful than a vaccine in both prevention and treatment since it is generally easier to manufacture, store,
that are from either the host or the virus itself. It has been shown that transmembrane protease serine 2 (TMPRSS2) can prime Spike for interactions with the human cell host receptor ACE2 during the virus entry process.\textsuperscript{11} Cathepsin L (CtsL) also potentiates the membrane fusion between SARS-CoV-2 with the endosome in infected cells.\textsuperscript{12} It has also been suggested that other cathepsins such as cathepsin B (CtsB) serve a role in the SARS-CoV-2 entry.\textsuperscript{13} After the SARS-CoV-2 genomic RNA is released into the host cytosol, it is translated by the host ribosome to form two large polypeptides, pp1a and pp1ab. The processing of pp1a and pp1ab to 16 nonstructural proteins (nsps) requires proteolytic functions of two internal protease fragments, nsp3 and nsp5 that are also called papain-like protease (PL\textsuperscript{pro}) and main protease (MPro), respectively. Some nsps package into an RNA replicase complex that replicates both genomic and subgenomic RNAs. The translation of subgenomic RNAs leads to essential structural proteins for packaging new virions. Furin is a host protease that can hydrolyze Spike to prime it for new virion packaging and release.\textsuperscript{14} Based on our current understanding of SARS-CoV-2 pathogenesis and replication, there are at least three host and two viral proteases serving critical roles in the SARS-CoV-2 life cycle. Inhibition of any of these enzymes will potentially cause a strong antiviral effect. The catalytic similarity between these enzymes makes it possible that a developed inhibitor is unsuitable for these enzymes. MPro, PL\textsuperscript{pro}, CtsB, and CtsL are cysteine proteases with a similar catalytic mechanism. TMPRSS2 and furin are serine proteases. Although serine proteases are mechanistically different from cysteine proteases, many currently developed MPro inhibitors have covalent warheads such as aldehyde and ketone that are prone to form covalent adducts with TMPRSS2 and furin as well.\textsuperscript{15,16} All of these proteases are also localized in different parts of the host cell. Their inhibition requires different characteristics in inhibitors such as cellular permeability and pH sensitivity. A simple antiviral assay for an inhibitor will likely lead to a positive result that is not from the inhibition of MPro and therefore causes a misunderstanding that can be detrimental to further rounds of lead optimization. Therefore, an assay system that directly reflects MPro inhibition in the host cell is critical for both the assessment and optimization of MPro inhibitors. In the current work, we describe such a system and its application in the evaluation of a number of developed and repurposed MPro inhibitors.

### RESULTS

The Rationale and the Establishment of a Cellular MPro Inhibition Assay for MPI8. A typical antiviral assay for SARS-CoV-2 is its induced strong cytopathogenic effect (CPE) in host cells that can be quantified by counting viral plaques (Figure 1B). An MPro inhibitor with high cellular potency will suppress this virus-induced CPE and therefore lead to host cell survival. A good cellular MPro inhibition assay will need to provide results similar to this CPE suppression process. Our original design for a cellular MPro inhibition assay was to express MPro in host cells that is fused with an N-terminal cyan fluorescent protein (CFP) and a C-terminal yellow fluorescent protein (YFP) and characterize the inhibition of autocleavage of this fusion protein in the presence of an inhibitor. MPro natively cuts off its fused protein at the C-terminus. We put an MPro digestion site between CFP and MPro as well. CFP and YFP form a Förster resonance energy transfer (FRET) pair.\textsuperscript{17} Without an inhibitor, both CFP and YFP will be cleaved from the fusion protein in host cells, which leads to no FRET signal. In the presence of a potent inhibitor, the fusion protein will be intact and emit strong FRET signals. However, transfection of 293T cells with pECFP-MPro-EYFP (Figure S1), a plasmid containing a gene coding the CFP-MPro-YFP fusion protein led to the death of most transfected cells (Figure S2). Repeating this transfection process all led to the exact same result. It is evident that MPro can exert acute toxicity to its human cell host. The same observation has been made by others and used to develop assays as well.\textsuperscript{18} MPI8 is an MPro inhibitor that our lab developed previously.\textsuperscript{15} Antiviral analysis indicated that MPI8 has potency to totally suppress SARS-CoV-2-induced
CPE in ACE2+ A549 cells at 0.2 μM. Given its approved antiviral potency, we used MPI8 as a positive control molecule for the analysis of cellular MPro inhibition. To alleviate the toxicity that was induced by the expression of CFP-MPro-YFP, we cultured 293T cells that were transfected with pECFP-MPro-EYFP in media containing 10 μM MPI8. The presence of MPI8 reduced death of transfected cells sharply. Interestingly, the overall expressed fusion protein was also significantly improved, showing much enhanced, directly detected yellow fluorescence from YFP (Figure S2). This positive correlation between the expression of CFP-MPro-YFP and the survival of transfected cells is likely due to the shutting-down of translation by active MPro. Since the measurement of a cell survival-correlated fluorescence increase in the presence of an inhibitor is much simpler than the FRET characterization, we decided to adopt this new way to analyze the cellular potency of MPro inhibitors. To explore whether M Pro expression is correlated to the death of SARS-CoV-2-infected cells, we used SARS-CoV-2 WA1/2020 to infect both Vero E6 and ACE2+ A549 cells; a strong CPE was observed 12 h after infection, which correlated with strong MPro expression detected by Western blot (Figure S3).

Since a FRET system is not necessary, we modified our plasmid to express an MPro-eGFP fusion protein (Figure 1C) in host cells that can be easily analyzed using fluorescent flow cytometry. The expression of MPro-eGFP in host cells will trigger cell death that leads to weak fluorescence. This process can be reversed by adding a potent inhibitor with cellular activity. In order to use eGFP fluorescence to accurately represent expressed MPro, we introduced a Q306G mutation in MPro to abolish its cleavage of the C-terminal eGFP. MPro requires a free N-terminal serine for strong activity. To achieve this, we built two constructs as shown in Figure 2A and Figure S4. The first construct pLVX-MPro-eGFP-1 encodes MPro-eGFP with an N-terminal methionine that relies on host methionine aminopeptidases for its cleavage. The second construct pLVX-MPro-eGFP-2 encodes MPro-eGFP containing a short N-terminal peptide that has an MPro cleavage site at the end for its autocatalytic release. The transfection of 293T cells with two constructs showed that pLVX-MPro-eGFP-2 led to more potent toxicity to cells, and this toxicity was effectively
suppressed when we provided 10 μM MPI8 in the growth media (Figure 2B). Therefore, we selected pLVX-MPro-eGFP-2 for all of our following studies. We have noticed that 72 h provided optimal fluorescence detection indicating a slow turnover of MPro-eGFP. To demonstrate that cellular fluorescence is positively correlated to the concentration of the provided MPI8, we transfected 293T cells with pLVX-MPro-eGFP-2, grew transfected cells in the presence of four MPI8 concentrations (0, 20, 40, and 160 nM) for 72 h, and then sorted cells using fluorescent flow cytometry (Figure 2C). Both the number and intensity of fluorescent cells (FL1-A signal, >1 × 10^6) were positively dependent on the provided MPI8 concentration, indicating the feasibility of using the system to characterize the cellular potency of an MPro inhibitor. To demonstrate this feasibility, we transiently transfected 293T cells with pLVX-MPro-eGFP-2 and grew transfected cells in the presence of a cascade of MPI8 concentrations between 0.001 and 10 μM. After 72 h, we sorted cells according to their eGFP fluorescence intensity. Cells with an FL1-A signal above 1 × 10^6 were analyzed. We built a MATLAB script to calculate the average eGFP fluorescence intensity of all analyzed cells and plotted the average eGFP fluorescence intensity against the MPI8 concentration as shown in Figure 2D. The data showed obvious MPI8-induced saturation of MPro-eGFP expression and fitted nicely to a three-parameter dose-dependent inhibition mechanism in GraphPad Prism 9 for IC50 determination. The

Figure 3. Structures of inhibitors that were evaluated in their cellular inhibition of MPro. (A) Reversible covalent inhibitors designed for MPro. (B) Investigational covalent inhibitors that were developed for other targets. (C) Inhibitors that were identified via high-throughput screening. (D) FDA-approved medications that have been explored as MPro inhibitors. (E) Diaryl esters that have high potency to inhibit MPro.

Figure 4. Cellular potency of literature-reported MPro inhibitors. K777 is included as a potential MPro inhibitor.
Table 1. Determined Enzymatic and Cellular IC₅₀ Values in Inhibiting SARS-CoV-2 MPro for Different Inhibitors

| compound ID | enzymatic IC₅₀ (µM) | cellular IC₅₀ (µM) | cellular IC₅₀ (µM) with CP-100356 | antiviral EC₅₀ (µM) | compound ID | enzymatic IC₅₀ (µM) | cellular IC₅₀ (µM) | cellular IC₅₀ (µM) with CP-100356 | antiviral EC₅₀ (µM) |
|-------------|----------------------|-------------------|---------------------------------|------------------|-------------|----------------------|-------------------|---------------------------------|------------------|
| MPI1³⁵      | 0.100 ± 0.023        | >10               | >2                              | >5               | MG-132      | 3.9 ± 1.0³⁵         | 3.0 ± 0.2²⁴        | n.d.                           |                  |
| MPI2³⁵      | 0.103 ± 0.014        | >2                | >2                              | >5               | calpain inhibitor II³⁶ | 0.97 ± 0.27     | >10                | 2.07 ± 0.76³⁴         |                  |
| MPI3³⁵      | 0.0085 ± 0.0015      | >2                | >2                              | >5               | calpain inhibitor XII | 0.45 ± 0.06³⁶     | >10                | 0.49 ± 0.18³⁶         |                  |
| MPI4³⁵      | 0.015 ± 0.005        | >2                | 1.8 ± 0.01                      | >5               | K777²²       | >100                | n.d.               | 0.62³⁶              |                  |
| MPI5³⁵      | 0.033 ± 0.002        | 0.66 ± 0.15       | 0.58 ± 0.06                     | 0.073 ± 0.007    | carmofur    | 1.35 ± 0.04³⁶       | >100               | n.d.               |                  |
| MPI6³⁵      | 0.060 ± 0.004        | 0.12 ± 0.03       | 0.075 ± 0.008                   | 0.21 ± 0.02      | tideguslin⁷  | 1.55 ± 0.30         | n.d.               | >100               |                  |
| MPI7³⁵      | 0.047 ± 0.003        | 0.19 ± 0.03       | 0.075 ± 0.006                   | 0.17 ± 0.02      | ebselen⁷     | 0.67 ± 0.09         | n.d.               | 4.67 ± 0.80³⁶        |                  |
| MPI8³⁵      | 0.105 ± 0.022        | 0.031 ± 0.002     | 0.039 ± 0.007                   | 0.030 ± 0.003    | disulfiram⁷  | 9.35 ± 0.18         | n.d.               |                  |                  |
| MPI9³⁵      | 0.056 ± 0.014        | >2                | >2                              | 3.37 ± 1.68³⁶/16/0.70²⁰ | bepridil²¹ | 72 ± 3               | n.d.               | 0.46³⁶              |                  |
| GC376       | 0.030 ± 0.0086³⁵     | >2                | 2.2 ± 0.2                       | 3.53 ± 0.01⁴⁴   | chloroquine²⁵,³¹ | 3.9 ± 0.2 >10⁴     | n.d.               | 5.47³⁸              |                  |
| 11a         | 0.053 ± 0.005,³⁴     | >2                | 1.4 ± 0.1                       | 1.31 ± 0.58³⁶/16/15.57²⁰ | hydroxychloroquine²⁵,³¹ | 2.9 ± 0.3 >10⁴   | n.d.               | 0.72³⁸              |                  |
| boceprevir  | 4.2 ± 0.6³⁶/8.0 ± 1.5³⁰ | >10              | 7.3 ± 2.3³⁶/7.3 ± 2.3³⁶/8.0 ± 1.5³⁰ | 1.31 ± 0.58³⁶/16/15.57²⁰ | hydroxychloroquine²⁵,³¹ | 2.9 ± 0.3 >10⁴   | n.d.               | 0.72³⁸              |                  |
| telaprevir²¹| 15.3                 | >10               | 10.1                            | 0.040 ± 0.004    | >10         | >10                 | >10               |                   |                  |
| calpeptin²⁶ | 10.7 ± 2.8           | n.d.              | 10.2                            | 0.068 ± 0.005    | >10         | >10                 | >10               |                   |                  |
| MG-115      | 3.1 ± 1.0³⁶/2.7 ± 0.1³⁶ | n.d.              | 10.3                            | 5.72 ± 0.43      | >10         | >10                 | >10               |                   |                  |

*Primary CPE assay. *Genomic RNA quantification. *Toxic at 10 µM. *Determined separately by us. *n.d.: not detected.
determined cellular IC_{50} value of MPI8 is 31 nM. To confirm that cell survival was from the direct inhibition of M^{Pro} protease activity by MPI8, we constructed pLVX-M^{Pro}(C145S)-eGFP whose encoded M^{Pro} has its active site cysteine mutated to serine. Transfecting 293T cells with this construct led to strong M^{Pro}(C145S)-eGFP expression that was detected by both a fluorescence measurement and Western blot and low cell death regardless of whether 1 μM MPI8 was present (Figures S5 and S6). Similarly, an M^{Pro} targeting siRNA significantly reduced cellular apoptosis in 293T cells that transiently expressed M^{Pro}-eGFP (Figure S7). These results confirmed that host cell death was due to the protease activity of M^{Pro}. To confirm that MPI8 does not inhibit caspases that serve functions in apoptosis, we used antimycin A to induce apoptosis in 293T cells and cultured treated cells with or without 1 μM MPI8. In both conditions, the detected apoptosis levels were not significantly different (Figure S8). Collectively, these results confirm that MPI8 directly inhibits the protease activity of M^{Pro} in 293T cells to cause overall cell survival and overexpression of M^{Pro}-eGFP.

Since MPI8 is highly effective in inhibiting M^{Pro} in cells, we used it in combination with pLVX-M^{Pro}-eGFP-2 to make stable 293T cells that continuously expressed M^{Pro}-eGFP. Using this stable cell line, we characterized M^{Pro}-induced apoptosis that was detected by annexin. After we withdrew MPI8 from the growth media, a strong apoptotic effect started to show after 24 h and continued to increase (Figure S9). Since MPI8 is a reversible covalent inhibitor, the relatively long incubation time for the observation of apoptosis is likely due to its slow release from the M^{Pro} active site. Due to concerns about residual MPI8 and its potential slow release from M^{Pro} in stable cells, we chose to perform a cellular potency characterization of all M^{Pro} inhibitors by performing a transient transfection of 293T cells and then growth in the presence of different inhibitor concentrations.

MPI1–7, MPI9, GC376, and 11a. MPI8 was one of 9 β-(S-2-oxoproprylidin-3-yl)alanin (Opal)-based, reversible covalent M^{Pro} inhibitors (MPI1–9) that we previously developed (Figure 3A). GC376 is a produg that dissociates quickly in water to release its Opal component. 11a is another Opal-based, reversible covalent M^{Pro} inhibitor that was developed in 2020. All 11 compounds showed high potency in inhibiting M^{Pro} in an enzymatic assay. Besides MPI8, we tested the cellular potency of all other 10 Opal inhibitors in their cellular inhibition of M^{Pro} by following the exact same procedure that we did for MPI8. As shown in Figure 4A, all tested Opal inhibitors promoted cell survival and the expression of M^{Pro}-eGFP significantly at 10 μM. However, data collected at different concentrations showed that only three inhibitors, MPI5, MPI6, and MPI7, induced the saturation of M^{Pro}-eGFP expression at or below 10 μM. Determined IC_{50} values for MPI5, MPI6, and MPI7 are 0.66, 0.12, and 0.19 μM, respectively (Table 1). Based on the collected data, MPI2–4, MPI9, GC376, and 11a have IC_{50} values higher than 2 μM, and MPI1 has an IC_{50} value higher than 10 μM.

Bepridil, Chloroquine, and Hydroxychloroquine. Using computational docking analysis in combination with experimental examination to guide drug repurposing for COVID-19, we previously showed that bepridil, an antianginal drug, inhibited M^{Pro} and had high potency in inhibiting SARS-CoV-2 replication in host cells. To provide a full picture to understand the mechanism of bepridil in inhibiting SARS-CoV-2, we applied our cellular assay to bepridil. As shown in Figure 4D, bepridil displayed a very weak inhibition of M^{Pro} in cells up to 10 μM. A previous publication reported that chloroquine and hydroxychloroquine are potent inhibitors of M^{Pro}. We applied our cellular assay to these two drugs. At all tested concentrations, both drugs displayed a close to undetectable promotion of M^{Pro}-eGFP expression, which indicates very low M^{Pro} inhibition from both drugs in cells. Using both a commercial and homemade substrate, we recharacterized M^{Pro} enzymatic inhibition by chloroquine and hydroxychloroquine. Our data (Figure S11) showed that M^{Pro} retained 84% activity at 16 μM chloroquine, and hydroxychloroquine did not inhibit M^{Pro} up to 16 μM.

Diaryl Esters 10-1, 10-2, and 10-3. Benzotriazole esters that were contaminants in a peptide library were accidentally discovered as potent inhibitors of SARS-CoV M^{Pro}. Based on their inhibition mechanism, a number of diaryl esters were developed later as potent SARS-CoV M^{Pro} inhibitors. To show whether similar compounds will also inhibit M^{Pro} of SARS-CoV-2, we synthesized diaryl esters 10-1, 10-2, and 10-3 and characterized their enzymatic inhibition IC_{50} values as 0.067, 0.038, and 7.6 μM, respectively (Figure S12). Using our
cellular assay, we characterized all three compounds as well. As shown in Figure 4D, all three compounds display observable potency in inhibiting MPro to promote MPro-eGFP expression at 2 and 10 μM. Their cellular MPro inhibition IC50 values are estimated above 10 μM.

**Effect of CP-100356 on the Cellular Potency of Peptide-Based MPro Inhibitors.** CP-100356 is a high-affinity inhibitor of multidrug resistance protein (Mdr-1/gp), a prototypical ABC transport that exports toxic substances from the inside of cells. A previous report showed that CP-100356 enhanced the antiviral potency of MPro inhibitors significantly.28 To investigate whether CP-100356 improves the cellular MPro inhibition potency of Opal inhibitors, we recharacterized MPI1–9, GC376, and 11a using our cellular assay in the presence of 0.5 μM CP-100356 (Figure 5). Except for MPI8 that showed an inhibition curve in the presence of CP-100356 very similar to that in the absence of CP-100356 and had a determined IC50 value of 39 nM, all other Opal inhibitors displayed a better cellular MPro inhibition curve. MPI5 and MPI6 have IC50 values (580 and 75 nM, respectively) in the presence of CP-100356 that are slightly lower than those in the absence of CP-100356. The highest cellular potency improvement that we observed among all compounds was for MPI7. It displayed an IC50 value (75 nM) lower than those in the absence of CP-100356. The general trends of observed cellular IC50 values are similar to those in the absence of CP-100356.

**Determination of Antiviral EC50 Values for MPI1–8.** Our previous antiviral assay for Opal inhibitors was based on the on–off observation of a CPE in Vero E6 and ACE2+ A549 cells. To quantify antiviral EC50 values of MPI1–8, we conducted plaque reduction neutralization tests of SARS-CoV-2 in Vero E6 cells in the presence of MPI1–8. We infected Vero E6 cells with SARS-CoV-2, grew infected cells in the presence of different inhibitor concentrations for 3 days, and then quantified SARS-CoV-2 plaque reduction. Based on SARS-CoV-2 plaque reduction in the presence of MPI1–8, we determined antiviral EC50 values for MPI1–8 as shown in Figure 6 and Table 1. MPI1–4 displayed a low antiviral potency with estimated EC50 values above 5 μM, and MPI5–8 have EC50 values determined to be 0.073, 0.21, 0.17, and 0.030 μM, respectively.

**DISCUSSION**

MPI1–9 were previously developed as potent MPro inhibitors. All showed enzymatic IC50 values around or below 100 nM (Table 1). Among them, MPI3 has the highest potency with an IC50 value of 8.5 nM. However, a CPE-based antiviral assay in Vero E6 cells showed that MPI3 weakly inhibited SARS-CoV-2.25 On the contrary, MPI8 that has an enzymatic IC50 value of 105 nM displayed the highest potency in inhibiting SARS-CoV-2. A separate antiviral assay in ACE2+ AS49 cells showed that MPI8 inhibited the SARS-CoV-2-induced CPE completely at 200 nM MPI8. Overall, the antiviral potency of MPI1–9 based on the on–off observation of the CPE correlates with their cellular MPro inhibition potency that we have detected using the new cellular assay. In order to confirm that the determined cellular potency results correlate closely with antiviral effects, we quantified antiviral EC50 values for MPI1–8 in Vero E6 cells. Overall, the general trends of determined potency for MPI1–8 from two assays correlate well with each other, indicating that the developed cellular assay is valid in assessing the antiviral potency of MPro inhibitors if these inhibitors act on MPro alone. CP-100356 improved the cellular potency for most Opal-based inhibitors, although this improvement is as dramatic as reported in Hoffman et al.28 Therefore, the main reason for the low cellular and antiviral potency of MPI3 and other Opal-based inhibitors might not be their active exportation from cells. Possible reasons that may contribute to the low antiviral and cellular potency for these compounds include their potential low cell permeability and proneness to both extracellular and intracellular proteolysis. Although MPI8 is not the most potent Opal-based inhibitor according to its in vitro enzymatic inhibition potency, it has the best antiviral and cellular potency. Its determined cellular IC50 is 31 nM, which is less than a third of its in vitro enzymatic IC50 value. A likely reason is the possible accumulation of MPI8 in cells, which needs to be investigated. Other Opal-based inhibitors with high cellular potency are MPI5, MPI6, and MPI7. All display cellular IC50 values below 1 μM. Among all 30 inhibitors that we have tested, MPI5–8 show the highest potency, which warrants their further investigation for possible use in treating COVID-19. As far as we know, MPI8 is the compound with the highest cellular MPro inhibition potency and the highest SARS-CoV-2 antiviral potency in Vero E6 cells. We urge its preclinical investigation for treating COVID-19.

GC376 is an investigational drug for treating feline infectious peritonitis, a lethal coronavirus disease in cats.19 Anivive Lifesciences Inc. did clinical investigations to repurpose GC376 for the treatment of COVID-19 patients. Although GC376 has high in vitro enzymatic MPro inhibition
potency with an IC_{50} value of 30 nM, it shows relatively weak cellular potency (IC_{50} > 2 μM). This weak cellular potency correlates with its antiviral potency that was determined with an EC_{50} value of 3.37 or 0.7 μM from two separate studies.\textsuperscript{16,20} In comparison to MPI8, GC376 is almost 2 orders of magnitude less potent in cellular and antiviral potency. A low cellular permeability and stability likely contribute to this low cellular and antiviral potency. 11a is an M_{Pro} inhibitor that has an antiviral EC_{50} value of 0.53 μM which is not significantly different from those of MPI6 and MPI7.\textsuperscript{9} However, its cellular potency is much weaker compared to MPI6 and MPI7. Its estimated cellular IC_{50} value is higher than 2 μM. It is likely that 11a may interfere with other critical process(es) in the SARS-CoV-2 life cycle to exert a potent antiviral effect, which needs to be explored.

Boceprevir and telaprevir are two drugs approved for treating hepatitis C virus infection. Both have shown potency to inhibit M_{Pro} enzymatically, and boceprevir has also been characterized in an antiviral assay to show an EC_{50} value of 1.31 μM.\textsuperscript{16} However, both drugs display very weak potency in their cellular M_{Pro} inhibition tests. Since we detected very weak cellular potency for boceprevir at 10 μM, boceprevir must hit on other key step(s) in the SARS-CoV-2 pathogenesis and replication pathway to convene its high antiviral effect. An investigation in this possibility will likely lead to the discovery of novel target(s) for COVID-19 drug development. Other aldehyde- and ketone-based inhibitors we have tested include calpeptin, MG-132, MG-115, calpain inhibitor II, and calpain inhibitor XII. Except for calpain inhibitor XII that showed a weak cellular inhibition of M_{Pro} with an estimated IC_{50} value higher than 10 μM, all others exhibited a close to undetectable M_{Pro} inhibition in cells up to 10 μM. Both calpain inhibitor II and XII have demonstrated antiviral potency toward SARS-CoV-2 with an EC_{50} value of 2.07 and 0.49 μM, respectively. Based on our cellular potency analysis of the two compounds, it is clear that their antiviral potency is not primarily from the inhibition of M_{Pro}. Wang et al. have explored compounds with dual functions to inhibit both M_{Pro} and host calpains/cathepsins as antivirals for SARS-CoV-2.\textsuperscript{29} These compounds include calpain inhibitor II and XII. As such, they likely inhibit host proteases to cause potent antiviral effects. K777 weakly inhibited M_{Pro} in a kinetic assay but potently inhibited SARS-CoV-2 in an antiviral assay.\textsuperscript{25} It showed undetectable potency in our cellular assay, which confirms that it must target other key process(es) in the SARS-CoV-2 life cycle.

Carfomur, tidaglusib, ebselen, disulfram, and PX-12 were discovered as M_{Pro} inhibitors from high-throughput screening. Although carfomur has an enzymatic IC_{50} value of 1.35 μM and generates a permanent covalent adduct with the M_{Pro} active site cysteine by forming a thio carbamate, it showed undetectable cellular potency up to 10 μM in our cellular assay. This observation correlates well with its low antiviral potency.\textsuperscript{23} The high chemical reactivity of carfomur likely contributes to its low cellular and antiviral potency. Tidaglusib, ebselen, disulfram, and PX-12 are redox-active compounds that can form covalent adducts with the M_{Pro} active site cysteine. Except for PX-12 that showed weak cellular potency at 10 μM, the other three drugs exhibited undetectable cellular potency up to 10 μM. Among the four compounds, only ebselen has been examined in an antiviral assay.\textsuperscript{7} It has a determined EC_{50} value of 4.67 μM. Since ebselen showed undetectable cellular M_{Pro} inhibition up to 10 μM, its antiviral potency must be from its interference with other key process(es) in the SARS-CoV-2 life cycle. The revelation of the SARS-CoV-2 inhibition mechanism by ebselen will likely lead to the discovery of novel drug target(s) for COVID-19.

Bepridil is an M_{Pro} inhibitor with an enzymatic IC_{50} value of 72 μM but a much lower antiviral EC_{50} value of 0.46 μM in ACE2+ A549 cells. Bepridil is known to inhibit other human viral pathogens as well.\textsuperscript{30} We detected a close to undetectable cellular M_{Pro} inhibition potency for bepridil up to 10 μM. This correlates with its relatively high enzymatic IC_{50} value. Therefore, bepridil must use a mechanism different from the inhibition of M_{Pro} to convene its high antiviral potency. This needs to be investigated. Chloroquine and hydroxychloroquine are two repurposed drugs for COVID-19 with demonstrated antiviral EC_{50} values of 5.47 and 0.72 μM, respectively.\textsuperscript{31} Although TMPRSS2 was shown as a possible target of chloroquine and hydroxychloroquine,\textsuperscript{32} a previous report showed that chloroquine and hydroxychloroquine potently inhibited M_{Pro} in an enzyme inhibition assay.\textsuperscript{25} We tested both drugs using the new cellular assay but revealed close to undetectable cellular M_{Pro} inhibition up to 10 μM for both drugs. We recharacterized the enzymatic inhibition of M_{Pro} by both drugs as well. However, we were not able to detect any M_{Pro} inhibition by hydroxychloroquine up to 16 μM, and chloroquine exhibited very weak inhibition of M_{Pro} at 16 μM. Based on our cellular data, enzymatic inhibition data, and data from a separate study,\textsuperscript{23} we are confident that both chloroquine and hydroxychloroquine do not potently inhibit M_{Pro}. Their antiviral activities are from different mechanism(s).

10-1, 10-2, and 10-3 are three diaryl esters in which 10-1 and 10-2 displayed high potency in inhibiting M_{Pro} enzymatically. All three compounds displayed a significant cellular M_{Pro} inhibition potency at 10 μM, but their potency is much lower than those of MPI5–8. Although 10-3 has a much weaker enzymatic inhibition potency than 10-1 and 10-2, its cellular potency is slightly better than those from 10-1 and 10-2. A likely explanation is that 10-3 is more stable than 10-1 and 10-2, which leads to a longer cellular time to convene its cellular M_{Pro} inhibition potency. Therefore, we recommend balancing cellular stability and enzymatic inhibition potency for the future development of diaryl esters as M_{Pro} inhibitors to achieve optimal antiviral effects.

As a prototypical ABC transporter inhibitor, CP-100356 can potentially improve the intracellular accumulation of exogenous toxic molecules in cells. Providing CP-100356 improved the cellular activity for all Opal-based inhibitors except MPI8, albeit the improvement is not as great as what was reported for PF-00835231.\textsuperscript{28} This is likely due to a low expression of Mdr-1/gp in 293T cells. Since CP-100356 is not an approved drug, its use in combination with an M_{Pro} inhibitor for COVID-19 treatment will face significant hurdles in clearing out toxicity and other clinical concerns. MPI8 showed a similar cellular potency in the presence and absence of CP-100356, suggesting MPI8’s high propensity to accumulate inside cells. This explains our observation that the determined cellular M_{Pro} inhibition IC_{50} value for MPI8 was 3-fold less than its determined enzymatic inhibition IC_{50} value. Data related to the use of CP-100356 support that MPI8 is optimal for cellular M_{Pro} inhibition. As the compound with the highest cellular and antiviral potency among all the literature and new compounds that we have tested in the current study, we urge MPI8 for further investigations in treating COVID-19.
CONCLUSION

We have developed a cellular assay to determine the cellular potency of SARS-CoV-2 M\textsuperscript{pro} inhibitors. Unlike an antiviral assay in which the interference of any key step in the SARS-CoV-2 life cycle may lead to a strong antiviral effect, this new cellular assay reveals only cellular M\textsuperscript{pro} inhibition potency of a compound. It provides precise information that reflects real M\textsuperscript{pro} inhibition in cells. Using this assay, we characterized 30 M\textsuperscript{pro} inhibitors. Our data indicated that 11a, boceprevir, ebselen, calpain inhibitor II, calpain inhibitor XII, K777, and bepridil likely interfere with key processes other than the M\textsuperscript{pro} catalysis in the SARS-CoV-2 pathogenesis and replication pathways to convene their strong antiviral effects. Our results also revealed that MPI8 has the highest cellular potency among all compounds that were tested. It has a cellular M\textsuperscript{pro} inhibition IC\textsubscript{50} value of 31 nM. MPI8 has been recently shown with dual MPro inhibition in cells. Using this assay, we characterized 30 compounds. It provides precise information that reflects real M\textsuperscript{pro} inhibition IC\textsubscript{50} value of 31 nM. MPI8 has been recently shown.

METHODS

Chemicals, Reagents, and Cell Lines from Commercial Providers. We purchased HEK293T/17 cells from ATCC; DMEM with high glucose with GlutaMAX supplement, fetal bovine serum, 0.25% trypsin-EDTA, phenol red, puromycin, Lipofectamine 3000, and dimethyl sulfoxide from Thermo Fisher Scientific; linear polyethyleneimine MW 25000 from Polysciences; RealTime-Glo annexin V apoptosis and a necrosis assay kit from Promega; an EndoFree plasmid DNA midi kit from Omega Biotek; antymycin a from Sigma-Aldrich; GC376 from Selleck Chem; boceprevir, calpeptin, MG-132, telaprevir, and carmofur from MedChemExpress; ebselen from RAM, telaprevir, and carmofur from MedChemExpress; ebselen from Cayman Chemical; chloroquine diphosphate from Alfa Aesar; hydroxylchloroquine sulfate from Acros Organics; and a fluorogenic M\textsuperscript{pro} substrate DABCYL-Lys-D-Ala-D-Ala-D-Ile-D-val-Met-AMC from Bachem. K777 was a gift from Prof. Thomas Meek at Texas A&M University. The synthesises of MPI1–9 and 11a were shown in a previous publication.\textsuperscript{15}

Plasmid Construction. We amplified M\textsuperscript{pro} with an N-terminal KTSAVLQ sequence using primers FRET-M\textsuperscript{pro}-for and FRET-M\textsuperscript{pro}-rev (Table S1) and cloned it into the pECFP-18aa-EYFP plasmid (Addgene, 109330) between XhoI and HindIII restriction sites to afford pECFP-M\textsuperscript{pro}-EYFP. To construct pLVX-M\textsuperscript{pro}-eGFP-1, we amplified M\textsuperscript{pro} with an N-terminal methionine using primers XbaI-M\textsuperscript{pro}-f and M\textsuperscript{pro}-HindIII-r (Table S1) and eGFP using primers HindIII-eGFP-f and eGFP-NotI-r. We digested the M\textsuperscript{pro} fragment using XbaI and HindIII-HF restriction enzymes and the eGFP fragment using HindIII-HF and NotI restriction enzymes. We ligated the two digested fragments together with the pLVX-EF1a-IRES-Puro vector (Takara Bio 631988) that was digested at XbaI and NotI restriction sites. To facilitate the ligation of these fragments, we used a ratio of M\textsuperscript{pro}, eGFP, and pLVX-EF1a-IRES-Puro digested products of 3:3:1. We constructed pLVX-M\textsuperscript{pro}-eGFP-2 in the same way as pLVX-M\textsuperscript{pro}-eGFP-1 except that we amplified the M\textsuperscript{pro} fragment using primers XbaI-CutMpro-f and M\textsuperscript{pro}-HindIII-r (Table S1). XbaI-Cut-M\textsuperscript{pro}-f encodes an MKTSAVLQ sequence for its integration to the M\textsuperscript{pro} N-terminus. To construct pLVX-M\textsuperscript{pro}C145S-eGFP, two primers M\textsuperscript{pro}C145Sf and M\textsuperscript{pro}C145S-r (Table S1) were used to carry out a site-directed mutagenesis of pLVX-M\textsuperscript{pro}-eGFP. All plasmids were sequence confirmed by Sanger sequencing.

Transfection and MPI8 Inhibition Tests Using pECFP-M\textsuperscript{pro}-EYFP. We grew 293T cells to 60% confluency and then transfected them with pECFP-M\textsuperscript{pro}-EYFP using Lipofectamine 3000. We added 10 µM MPI8 at the same time of transfection. After 72 h of incubation, cells were collected and analyzed by a flow cytometer as well as fluorescence microscopy. In order to obtain high-definition images, poly-d-lysine-coated glass bottom plates from Mattek were used for microimaging.

Transfection and Inhibition Tests Using pLVX-M\textsuperscript{pro}-eGFP-1 and pLVX-M\textsuperscript{pro}-eGFP-2. We grew 293T cells to 60% confluency and transfected them with pLVX-M\textsuperscript{pro}-eGFP-1 or pLVX-M\textsuperscript{pro}-eGFP-2 using Lipofectamine 3000. We added different concentrations of MPI8 from the nM to µM level at the same time of transfection. After 72 h of incubation, we analyzed the transfected 293T cells using flow cytometry to determine fluorescence cell numbers and the eGFP fluorescence intensity.

Establishment of 293T Cells Stably Expressing M\textsuperscript{pro}-eGFP. To establish a 293T cell line that stably expresses M\textsuperscript{pro}-eGFP, we packaged lentivirus particles using the pLVX-M\textsuperscript{pro}-eGFP-2 plasmid. Briefly, we transfected 293T cells at 90% confluency with three plasmids including pLVX-M\textsuperscript{pro}-eGFP-2, pMD2.G, and psPAX2 using 30 µg/mL polyethylenimine. We collected supernatants at 48 and 72 h after transfection separately. We concentrated and collected lentiviral particles from collected supernatant using ultracentrifugation. We then transduced fresh 293T cells using the collected lentivirus particles. After 48 h of transduction, we added puromycin to the culture media to a final concentration of 2 µg/mL. We gradually raised the puromycin concentration 10 µg/mL in 2 weeks. The final stable cells were maintained in media containing 10 µg/mL puromycin.

Apoptosis Analysis. We performed the apoptosis analysis of the M\textsuperscript{pro} stable cells and cells transiently transfected with the pLVX-M\textsuperscript{pro}-eGFP-2 plasmid using the RealTime-Glo annexin V apoptosis and necrosis assay kits from Promega. The cells were maintained in a high glucose DMEM medium supplemented with 10% fetal bovine serum (FBS), plated with a cell density of 5 × 10\textsuperscript{5} cells/mL. We set up five groups of experiments including (1) HEK 293T/17, (2) HEK 293T/17 + MPI8 (1 µM), (3) HEK 293T/17 cells stably expressing M\textsuperscript{pro}-eGFP, (4) HEK 293T/17 cells stably expressing M\textsuperscript{pro}-eGFP + MPI8 (1 µM), and (5) HEK 293T/17 or HEK 293T/17 cells stably expressing M\textsuperscript{pro}-eGFP + antimycin A (1 µM). Each experiment was repeated 5 times. The assay was performed according to the instructor's protocol. Chemiluminescence was recorded at 12, 24, 36, 48, 60, and 72 h after plating the cells. The luminescence readings were normalized using HEK 293T/17 as a negative control.

Cellular M\textsuperscript{pro} Inhibition Analysis for 29 Selected Compounds. We grew HEK 293T/17 cells in high-glucose DMEM with GlutaMAX supplement and 10% fetal bovine serum in 10 cm culture plates under 37 °C and 5% CO\textsubscript{2} to ~80–90% and then transfected cells with the pLVX-M\textsuperscript{pro}-eGFP-2 plasmid. For each transfection, we used 30 µg/mL polyethylenimine and a total of 8 µg of the plasmid in 500 µL of the opti-MEM medium. We incubated cells with transfecting reagents overnight. On the second day, we removed the
medium, washed cells with a PBS buffer, digested them with 0.05% trypsin-EDTA, resuspended the cells in the original growth media, adjusted the cell density to 5 × 10⁶ cells/mL, provided 500 µL of suspended cells in the growth media to each well of a 48-well plate, and then added 100 µL of a drug solution to the growth media. These cells were then incubated under 37 °C and 5% CO₂ for 72 h before their flow cytometry analysis.

**Data Collection, Processing, and Analysis.** The cell was incubated with various concentrations of drugs at 37 °C for 3 days. After 3 days of incubation, we removed the media and then washed the cells with 500 µL of PBS to remove dead cells. Cells were then trypsinized and spun down at 800 rpm for 5 min. We removed the supernatant and suspended the cell pellets in 200 µL of PBS. The fluorescence of each cell sample was collected by a CytoBeads in 200 µL of PBS. We removed the supernatant and suspended the cell pellets in 200 µL of PBS. The fluorescence of each cell sample was collected by a CytoBeads (SSC-A and SSC-H) and forward scattering (FSC-A). We gated cells based on SSC-A and FSC-A and then with SSC-A and SSC-H. The eGFP fluorescence was recorded by a blue laser (488 nm), and cells were collected at FITC-A (525 nm). After collecting the data, we analyzed and transferred the data to csv files containing information on each cell sample. We then analyzed these files using a self-written MATLAB program for massive data processing. We sorted the FITC-A column from smallest to largest. A 10th cutoff was set to separate the column into two groups, larger as positive and smaller as negative. We integrated the positive group and divided the total integrated fluorescence intensity by the total positive cell counts as Flu. Int. shown in all graphs. The standard deviation of positive fluorescence was also calculated. It was then plotted and fitted nonlinearly with an agonist curve (three parameters) against drug concentrations in the program Prism 9 from GraphPad for IC₅₀ determination.

**Kinetic Recharacterization of Chloroquine and Hydroxychloroquine.** We prepared 10 mM stock solutions of hydroxychloroquine and chloroquine in a PBS buffer and carried out IC₅₀ assays for both hydroxychloroquine and chloroquine by measuring activities of 50 nM MPro against a concentration range of 0–16 µM hydroxychloroquine and chloroquine. Serial dilutions of hydroxychloroquine and chloroquine were carried out in the assay buffer by keeping the PBS concentration the same. First, 100 nM MPro in the assay buffer (10 mM phosphate, 10 mM NaCl, 0.5 mM EDTA, pH 7.6) was treated with 2 times the working concentration of hydroxychloroquine and chloroquine at 37 °C for 30 min. Then, a 20 µM concentration of the fluorogenic MPro substrate Suh3 (prepared from a 1 mM stock solution of the dye in DMSO) in the assay buffer was added to the reaction mixture to a final concentration of 10 µM. Immediately after the addition of the substrate, we started to monitor the reaction in a BioTek Neo2 plate reader with an excitation wavelength at 336 nm and emission detection at 490 nm. Initial product formation slopes at the first 5 min were calculated by simple linear regression, and data were plotted in GraphPad Prism 9.

**Synthesis of 5-Chloropyridin-3-yl 1H-Indole-7-carboxylate (10-1).** To a solution of 5-chloropyridin-3-ol (1 mmol, 130 mg) and 1H-indole-7-carboxylic acid in anhydrous dichloromethane (DCM), we added DMAP (0.1 mmol, 12 mg) and EDC (1.2 mmol, 230 mg). The resulting solution was stirred at room temperature overnight. Then, the reaction mixture was evaporated in vacuo, and the residue was purified by flash chromatography to afford 10-1 as a white solid (190 mg, 69%).

**Kinetic Characterization of 10-1, 10-2, and 10-3 in Inhibiting MPro.** We performed MPro inhibition assays of 10-1, 10-2, and 10-3 according to the same procedure used for the kinetic characterization of hydroxychloroquine and chloroquine.

**Characterization of the Cellular Potency of MPI1–9, GC376, 11a, 10-1, 10-2, and 10-3 in the presence of CP-100356.** All cellular MPro inhibition assays for these 14 compounds were repeated with the addition of CP-100356 in DMSO to a final concentration of 0.5 µM. The overall assay process and analysis were identical to the assays without CP-100356.

**Plaque Reduction Neutralization Tests of SARS-CoV-2 by MPI5–8.** We seeded 18 × 10⁴ Vero cells per well in flat-bottomed 96-well plates in a total volume of 200 µL of a culturing medium (DMEM + 10% FBS + glutamine) and incubated cells overnight at 37 °C and under 5% CO₂. The next day, we titrated compounds in separate round-bottom 96-well plates using the culturing medium. We then discarded the original medium used for cell culturing and replaced it with 50 µL of compound-containing media from round-bottom plates. We incubated cells for 2 h at 36 °C and under 5% CO₂. After incubation, we added 1000 PFU/50 µL of SARS-CoV-2 (USA-WA1/2020) to each well and incubated the plate at 36 °C and under 5% CO₂ for 1 h. After incubation, we added 100
µL of overlay (1:1 of 2% methylcellulose and the culture medium) to each well. We incubated plates for 3 days at 36 °C and under 5% CO₂. Staining was performed by discarding the supernatant, fixing the plates with 4% paraformaldehyde in the PBS buffer for 30 min, and staining with crystal violet. Plaques were then counted.

**Safety Statement.** No unexpected or unusually high safety hazards were encountered.

### ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.1c00910.

Additional methods, figures, and tables including cellular toxicity results, plasmid maps, and fluorescence and recharacterization results (PDF)

### Accession Codes

The plasmid pLVX-M10-eGFP has been deposited to Addgene.

### AUTHOR INFORMATION

**Corresponding Authors**

Henry Ji — Sorrento Therapeutics, Inc., San Diego, California 92121, United States; Email: HJi@sorrentotherapeutics.com

Shiqing Xu — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States; orcid.org/0000-0001-6260-9290; Email: shiqing.xu@tamu.edu

Wenshe Ray Liu — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Department of Biochemistry and Biophysics, and Department of Molecular and Cellular Medicine, College of Medicine, Texas A&M University, College Station, Texas 77843, United States; Institute of Biosciences and Technology and Department of Translational Medical Sciences, College of Medicine, Texas A&M University, Houston, Texas 77030, United States; orcid.org/0000-0002-7078-6534; Email: wliu@chem.tamu.edu

**Authors**

Wenyue Cao — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Chia-Chuan Dean Cho — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Zhi Zachary Geng — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Namir Shaabani — Sorrento Therapeutics, Inc., San Diego, California 92121, United States

Xinyu R. Ma — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Erol C. Vatansever — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Yugendar R. Alugubelli — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Yuying Ma — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Sankar P. Chaki — Global Health Research Complex, Division of Research, Texas A&M University, College Station, Texas 77843, United States

William H. Ellenburg — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Kai S. Yang — Texas A&M Drug Discovery Laboratory, Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Robert Allen — Sorrento Therapeutics, Inc., San Diego, California 92121, United States

Benjamin W. Neuman — Department of Biology, Texas A&M University, College Station, Texas 77843, United States

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acscentsci.1c00910

**Author Contributions**

X.C.W., C.-C.D.C., Z.Z.G., and N.S. contributed equally to this work. W.R.L. conceived the project. W.C., C.-C.D.C., Z.Z.G., N.S., X.R.M., S.P.C., Y.Q., B.W.N., H.J., S.X., and W.R.L. designed and performed experiments. W.C., C.-C.D.C., Z.Z.G., N.S., S.X., and W.R.L. wrote the manuscript. All authors approved the final manuscript before submission.

**Notes**

The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

This work was supported by Welch Foundation (Grant A-1715 to W.R.L.), National Institute of Allergy and Infectious Diseases (Grant R21AI164088 to S.X.), the Texas A&M University President’s Excellence Fund to W.R.L., TAMU COS Strategic Transformative Research Program to S.X., and Texas A&M X Grants to W.R.L. and S.X. We thank Prof. Thomas Meek for providing us the compound K777.

### REFERENCES

(1) WHO. WHO Coronavirus Disease (COVID-19) Dashboard, 2021. https://covid19.who.int/ (accessed Dec 9, 2021).

(2) Forini, G.; Mantovani, A. Covid-19 Commission of Accademia Nazionale dei Lincei, R. COVID-19 vaccines: where we stand and challenges ahead. Cell Death Differ. 2021, 28 (2), 626–639.

(3) Morse, J. S.; Lalonde, T.; Xu, S.; Liu, W. R. Learning from the Past: Possible Urgent Prevention and Treatment Options for Severe Acute Respiratory Infections Caused by 2019-nCoV. ChemBioChem. 2020, 21 (5), 730–738.

(4) Lan, J.; Ge, J.; Yu, J.; Shan, S.; Zhou, H.; Fan, S.; Zhang, Q.; Shi, X.; Wang, Q.; Zhang, L.; et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. Nature 2020, 581 (7807), 215–220.

(5) Guruprasad, L. Human SARS CoV-2 spike protein mutations. Proteins 2021, 89 (5), 569–576.

(6) Jangra, S.; Ye, C.; Rathnasighe, R.; Stadlbauer, D.; Kramer, F.; Simon, V.; Martinez-Sobrido, L.; Garcia-Sastre, A.; Schotsaert, M. SARS-CoV-2 spike E484K mutation reduces antibody neutralisation. Lancet Microbe 2021, 2, e283.
antivirals against 3C or 3C-like proteases of picornaviruses, R.; Battaile, K. P.; Groutas, W. C.; Chang, K. O. Broad-spectrum (2016).

Sun, H.; Huang, B.; Niu, P.; et al. Both Boceprevir and GC376 Commun. of SARS-CoV-2 3CL protease inhibitors. Xing, L.; Stockwell, B. R.; et al. Lead compounds for the development F.; Liu, H.; Hong, S. J.; Lin, F. Y.; Nair, M. S.; Zask, A.; Huang, Y.; Cytotoxicity. ACS Chem. Biol.

He, C.; Chen, P. R. A Genetically Encoded FRET Sensor for 678

ACS Central Science http://pubs.acs.org/journal/acscii

2021

Research Article

2022, 8, 192–204

ACS Cent. Sci. 2022, 8, 192–204

119(44), 27381–27387.

Jung, S. H. An Overview of Severe Acute Respiratory Syndrome-CoV-2 main protease inhibitors. Bioorg. Med. Chem. Lett. 2008, 18 (20), 5684–5688.

Postnikova, E.; Kollins, E.; Alexander, I.; Gross, R.; Kleczkowska, K.; Li, L. Y.; et al. Discovery of Ketone-Based Covalent Inhibitors of Coronavirus 3CL Proteases for the Potential Therapeutic Treatment of COVID-19. J. Med. Chem. 2020, 63 (21), 12725–12747.

Sacco, M. D.; Ma, C.; Lagarias, P.; Gao, A.; Townsend, J. A.; Meng, X.; Dube, P.; Zhang, X.; Hu, Y.; Kitamura, N.; et al. Structure and inhibition of the SARS-CoV-2 main protease reveal strategy for developing dual inhibitors against M(pro). Nat. Commun. 2020, 11 (50), eabe0751.

DeWald, L. E.; Dyall, J.; Sword, J. M.; Torzewski, L.; Zhou, H.; Postnikova, E.; Kollins, E.; Alexander, I.; Gross, R.; Cong, Y.; et al. The Calcium Channel Blocker Bepridil Demonstrates Efficacy in the Murine Model of Marburg Virus Disease. J. Infect. Dis. 2018, 218 (55), S88–S99.

Yao, X.; Ye, F.; Zhang, M.; Cui, H.; Huang, B.; Niu, P.; Liu, X.; Zhao, L.; Dong, E.; Song, C.; et al. In Vivo Antiviral Activity and Projection of Optimized Dosing Design of Hydroxycloroquine for the Treatment of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Clin. Infect. Dis. 2020, 71 (15), 732–739.

Ou, T.; Mou, H.; Zhang, L.; Ojha, A.; Choe, H.; Farzan, M. Hydroxychloroquine-mediated inhibition of SARS-CoV-2 entry is attenuated by TMPRSS2. PLoS Pathog. 2021, 17 (1), e1009212.

Ma, C.; Wang, J. Dipyridamole, chloroquine, montelukast sodium, candesartan, oxytocycline, and atazanavir are not SARS-CoV-2 main protease inhibitors. Proc. Natl. Acad. Sci. U. S. A. 2021, 118 (8), e202440118.

Ma, X. R.; Alugubelli, Y. R.; Ma, Y.; Vatansever, E. C.; Scott, D. A.; Qiao, Y.; Yu, G.; Xu, S.; Liu, W. R. MP18 is Potent against SARS-CoV-2 by Inhibiting Dually and Selectively the SARS-CoV-2 Main protease. Nat. Commun. 2020, 11 (1), 4417.

Baker, J. D.; Uhrich, R. L.; Kraemer, G. C.; Love, J. E.; Kraemer, B. C. A drug repurposing screen identifies hepatitis C antivirals as inhibitors of the SARS-CoV2 main protease. PLoS One 2021, 16 (2), e0245962.

Mellott, D. M.; Tseng, C. T.; Drelich, A.; Fujitava, P.; Chernia, B. C.; Kostomiris, D. H.; Hsu, J.; Zhu, J.; Taylor, Z. W.; Kocurek, K. I.; et al. A Clinical-Stage Cysteine Protease Inhibitor blocks SARS-CoV-2 Infection of Human and Monkey Cells. ACS Chem. Biol. 2021, 16 (4), 642–650.

Jin, Z.; Zhao, Y.; Sun, Y.; Zhang, B.; Wang, H.; Wu, Y.; Zhu, Y.; Zhu, C.; Hu, T.; Du, X.; et al. Structural basis for the inhibition of SARS-CoV-2 main protease by antineoplastic drug carmofur. Nat. Struct. Mol. Biol. 2020, 27 (6), 529–532.

Vatansever, E. C.; Yang, K. S.; Drelich, A. K.; Kracht, K. C.; Cho, C. C.; Kempaiah, K. R.; Hsu, J. C.; Mellott, D. M.; Xu, S.; Tseng, C. K.; et al. Bepridil is potent against SARS-CoV-2 in vitro. Proc. Natl. Acad. Sci. U. S. A. 2021, 118 (10), e2012201118.

Li, Z.; Li, X.; Huang, Y. Y.; Wu, Y.; Liu, R.; Zhou, L.; Lin, Y.; Wu, D.; Zhang, L.; Liu, H.; et al. Identify potent SARS-CoV-2 main protease inhibitors via accelerated free energy perturbation-based virtual screening of existing drugs. Proc. Natl. Acad. Sci. U. S. A. 2020, 117 (44), 27381–27387.

Pillayyar, T.; Manickam, M.; Namasivayam, V.; Hayashi, Y.; Jung, S. H. A PLoS Pathog. 2020, 16 (11), e1009013.

He, C.; Chen, P. R. A Genetically Encoded FRET Sensor for the SARS coronavirus main proteinase by benzotriazole esters. J. Med. Chem. 2008, 51 (7), 12747–12759.

Blankenship, L. R.; et al. A Quick Route to Multiple Highly Potent SARS-CoV-2 Main Protease Inhibitors*. ChemMedChem. 2021, 16 (6), 942–948.

Cheng, Y. W.; Chao, T. L.; Li, C. L.; Chiu, M. F.; Kao, H. C.; Wang, S. H.; Pang, Y. H.; Lin, C. H.; Tsai, Y. M.; Lee, W. H.; et al. Furin Inhibitors Block SARS-CoV-2 Spike Protein Cleavage to Suppress Virus Production and Cytopathic Effects. Cell Rep. 2020, 33 (2), 108254.

Yang, K. S.; Ma, X. R.; Ma, Y.; Alugubelli, Y. R.; Scott, D. A.; Vatansever, E. C.; Drelich, A. K.; Sankaran, B.; Geng, Z. Z.; Blankenship, L. R.; et al. A Quick Route to Multiple Highly Potent SARS-CoV-2 Main Protease Inhibitors*. ChemMedChem. 2021, 16 (6), 942–948.

Ma, C.; Sacco, M. D.; Hurst, B.; Townsend, J. A.; Hu, Y.; Szeto, T.; Zhang, X.; Tarbet, B.; Marty, M. T.; Chen, Y.; et al. Development of a series of potent chloropyridyl ester-derived SARS-CoV 3CLpro inhibitors. Bioorg. Med. Chem. Lett. 2008, 18 (20), 5684–5688.

Hofmann, R. L.; Kania, R. S.; Brothers, M. A.; Davies, J. F.; Ferre, R. A.; Gaiwala, K. S.; He, M.; Hogan, R. J.; Kozminski, K.; Li, L. Y.; et al. Discovery of Ketone-Based Covalent Inhibitors of Coronavirus 3CL Proteases for the Potential Therapeutic Treatment of COVID-19. J. Med. Chem. 2020, 63 (21), 12725–12747.

Sacco, M. D.; Ma, C.; Lagarias, P.; Gao, A.; Townsend, J. A.; Meng, X.; Dube, P.; Zhang, X.; Hu, Y.; Kitamura, N.; et al. Structure and inhibition of the SARS-CoV-2 main protease reveal strategy for developing dual inhibitors against M(pro) and cathepsin L. J. Med. Chem. 2020, 63 (30), 678–692.

Song, Y.; Yang, M.; Wegner, S. V.; Zhao, J.; Zhu, R.; Wu, Y.; He, C.; Chen, P. R. A Genetically Encoded FRET Sensor for Intracellular Heme. ACS Chem. Biol. 2015, 10 (7), 1610–1615.

Resnick, S. J.; Iketani, S.; Hong, S. J.; Zask, A.; Liu, H.; Kim, S.; Melore, S.; Lin, F. Y.; Nair, M. S.; Huang, Y.; et al. Inhibitors of Coronavirus 3CL Proteases Protect Cells from Protease-Mediated Cytotoxicity. J. Virol. 2021, 95 (14), e0237420. Iketani, S.; Forouhar, F.; Liu, H.; Hong, S. J.; Lin, F. Y.; Nair, M. S.; Zask, A.; Huang, Y.; Xing, L.; Stockwell, B. R.; et al. Lead compounds for the development of a series of potent SARS-CoV-2 3CL protease inhibitors. Nat. Commun. 2021, 12 (1), 2016.

Kim, Y.; Lovell, S.; Tiew, K. C.; Mandadapu, S. R.; Alliston, K. R.; Battla, K. P.; Groutas, W. C.; Chang, K. O. Broad-spectrum antivirals against 3C or 3C-like proteases of picornaviruses, norroviruses, and coronaviruses. J. Virol. 2012, 86 (21), 11754–11762.

Fu, L.; Ye, F.; Feng, Y.; Yu, F.; Fang, Q.; Wu, Y.; Zhao, C.; Sun, H.; Huang, B.; Niu, P.; et al. Both Boceprevir and GC376 efficaciously inhibit SARS-CoV-2 by targeting its main protease. Nat. Commun. 2020, 11 (1), 4417.
Protease and the Host Cathepsin L.² ChemMedChem. 2022, 17 (1), e202100456.