Discovery of SARS-CoV-2 Papain-like Protease Inhibitors through a Combination of High-Throughput Screening and a FlipGFP-Based Reporter Assay

Chunlong Ma, Michael Dominic Sacco, Zilei Xia, George Lambrinidis, Julia Alma Townsend, Yanmei Hu, Xiangzhi Meng, Tommy Szeto, Mandy Ba, Xiujun Zhang, Maura Gongora, Fushun Zhang, Michael Thomas Marty, Yan Xiang, Antonios Kolocouris, Yu Chen,* and Jun Wang*

ABSTRACT: The papain-like protease (PLpro) of SARS-CoV-2 is a validated antiviral drug target. Through a fluorescence resonance energy transfer-based high-throughput screening and subsequent lead optimization, we identified several PLpro inhibitors including Jun9-72-2 and Jun9-75-4 with improved enzymatic inhibition and antiviral activity compared to GRL0617, which was reported as a SARS-CoV PLpro inhibitor. Significantly, we developed a cell-based FlipGFP assay that can be applied to predict the cellular antiviral activity of PLpro inhibitors in the BSL-2 setting. X-ray crystal structure of PLpro in complex with GRL0617 showed that binding of GRL0617 to SARS-CoV-2 induced a conformational change in the BL2 loop to a more closed conformation. Molecular dynamics simulations showed that Jun9-72-2 and Jun9-75-4 engaged in more extensive interactions than GRL0617. Overall, the PLpro inhibitors identified in this study represent promising candidates for further development as SARS-CoV-2 antivirals, and the FlipGFP-PLpro assay is a suitable surrogate for screening PLpro inhibitors in the BSL-2 setting.

INTRODUCTION

The COVID-19 pandemic has led to 170,812,850 confirmed cases and 3,557,586 deaths as of June 2, 2021, rendering it the worst pandemic since the 1918 Spanish flu. The etiological agent of COVID-19 is SARS-CoV-2, a single-stranded positive-sense RNA virus that belongs to the beta coronavirus genus. Two additional coronaviruses within the same genus, SARS-CoV and MERS-CoV, have caused epidemics in humans with mortality rates of 9.6% and 34.3%, respectively. Although SARS-CoV-2 has a lower mortality rate of 2.1% compared to SARS-CoV and MERS-CoV, it has led to a far greater death toll due to its higher transmission. SARS-CoV-2 differs from SARS-CoV and MERS-CoV in that it has a long incubation time after the initial infection (1–2 weeks), and a large percentage of infected patients continue to shed the virus while being asymptomatic, presenting a daunting task for surveillance and containment.

Two mRNA vaccines developed by Pfizer/BioNTech and Moderna and one adenovirus-based vaccine by Johnson and Johnson have been approved by FDA in the United States. For small molecule antivirals, remdesivir received FDA approval on October 22, 2020. Although the polymerase of SARS-CoV-2 has a proofreading function, it continues to mutate at a rate about 10⁻⁶ per site per cycle. Several variants have already emerged and have widely circulated among humans since the beginning of the pandemic. Therefore, there is a dire need for additional antivirals with a novel mechanism of action. Antivirals are not substitutes for vaccines but rather important complements that can be used for the treatment of infection from both wild-type (WT) and variant viruses. Among the viral proteins that have been actively pursued as important complements that can be used for the treatment of infection from both wild-type (WT) and variant viruses. Among the viral proteins that have been actively pursued as antiviral drug targets, the main protease (Mpro) and papain-like protease (PLpro) are the most promising ones. Mpro and PLpro are involved in the proteolytic digestion of the viral polyproteins pp1a and pp1ab, yielding individual functional viral proteins for the replication complex formation. PLpro cleaves at three sites with the recognition sequence "LXGG↓XX↓". PLpro has been shown to play additional roles in dysregulating the host immune response and impairing the host type I interferon antiviral effect through its deubiquitinating and deISG15ylating (interferon-induced gene 15)
activities, respectively.\textsuperscript{10–12} SARS-CoV-2 PL\textsuperscript{pro} cleaves ISG15 and polyubiquitin modifications from cellular proteins, and inhibition of PL\textsuperscript{pro} led to the accumulation of ISG15-conjugates and polyubiquitin conjugates.\textsuperscript{13} While SARS-CoV PL\textsuperscript{pro} prefers ubiquitinated substrates, SARS-CoV-2 PL\textsuperscript{pro} prefers the ISGylated proteins as substrates.\textsuperscript{10–12} PL\textsuperscript{pro} is part of a membrane -anchored multidomain protein named nonstructural protein 3 (nsp-3), an essential component of the replicate−transcriptase complex. The pleiotropic roles of SARS-CoV-2 PL\textsuperscript{pro} may make it a promising antiviral drug target. Substantial morbidity and mortality associated with COVID-19 infection is caused by cytokine storm\textsuperscript{14} and suppressing host immune response using dexamethasone and baricitinib has been shown to provide therapeutic benefits in the treatment of severe infections.\textsuperscript{15,16}

Notably, we developed the FlipGFP assay for quantifying the hits with sub-micromolar potency in the enzymatic assay. Subsequent lead optimization led to the discovery of several SARS-CoV-2 PL\textsuperscript{pro} inhibitors reported herein as promising hits for further development as SARS-CoV-2 PL\textsuperscript{pro} inhibitors. Using the FlipGFP-PLpro assay, suggesting that the FlipGFP-PLpro can be applied to faithfully predict the cellular antiviral activity of PL\textsuperscript{pro} inhibitors (Figure 2), had IC\textsubscript{50} values of 7.29 \pm 0.09 and 2.18 \pm 0.05 \mu M, respectively. \textsuperscript{12} PLpro is more efficient in cleaving the ubiquitin (Ub) and ISG15 (ISG) modifications than the viral polyprotein, with k\textsubscript{cat}/K\textsubscript{m} values of 1070 and 1.67 \times 10^5 M^{-1} s^{-1} (Table S2), respectively. This substrate preference is in agreement with results reported previously,\textsuperscript{24} and SARS-CoV PL\textsuperscript{pro} was also reported to have deubiquitinating and deISGylating activities.\textsuperscript{10–13,22} Accordingly, we characterized the deubiquitinating and deISGylating activities of SARS-CoV-2 PL\textsuperscript{pro}-His using the Ub-AMC and ISG-AMC substrates, respectively, in the enzymatic assay. It was found that SARS-CoV-2 PL\textsuperscript{pro}-His is more efficient in cleaving the ubiquitin (Ub) and ISG15 (ISG) modifications than the viral polyprotein, with k\textsubscript{cat}/K\textsubscript{m} values of 1070 and 1.67 \times 10^5 M^{-1} s^{-1} (Table S2), respectively. This substrate preference is in agreement with results reported previously,\textsuperscript{24} and SARS-CoV PL\textsuperscript{pro} was also reported to have a similar substrate preference.\textsuperscript{25} Significantly, the deISGylating activity is 156-fold higher than the deubiquitinating activity, which is consistent with previous reports that SARS-CoV-2 PL\textsuperscript{pro} prefers ISG15 over ubiquitin.\textsuperscript{9–13}

High-Throughput Screening of the Enamine 50K Diversity Library against the SARS-CoV-2 PL\textsuperscript{pro} and Hit Validation. The HTS assay was optimized in 384-well plates using the FRET substrate, which gave a Z' factor of 0.688 with a signal-to-noise ratio (S/B) of 11.2, indicating that this was a robust assay (Figure 1). We then performed the HTS against the enamine library, which consists of 50 240 structurally diverse compounds. GRL0617 was included as a positive control.
± 0.29 °C (Table S3), which is consistent with their enzymatic inhibition. In comparison, GRL0617 had an IC_{50} value of 2.05 ± 0.12 μM and increased the protein stability by 3.52 ± 0.27 °C in the DSF assay (Table S3). The potency of GRL0617 in inhibiting SARS-CoV-2 PL^{pro} from our study is consistent with recent reports.10−13 The rest of the hits had weak enzymatic inhibition (IC_{50} > 10 μM) and showed marginal binding to PL^{pro} (Table S3); therefore, they were not further pursued. Both Jun9-13-7 and Jun9-13-9 also inhibit the deubiquitinating and deISG Glyating activities with IC_{50} values ranging from 4.93 to 12.51 μM (Figure 2D and Table S4). In contrast, neither of these two compounds inhibited SARS-CoV-2 M^{pro} up to 200 μM (Figure S2), suggesting the inhibition of SARS-CoV-2 PL^{pro} is specific. The binding of Jun9-13-7 and Jun9-13-9 to SARS-CoV-2 PL^{pro} was further characterized using the native mass spectrometry (Figure 2E). It was shown that both Jun9-13-7 and Jun9-13-9 showed dose-dependent binding to PL^{pro} with binding stoichiometry of one drug per PL^{pro}, similar to the positive control GRL0617. Enzymatic kinetic studies showed that compounds Jun9-13-7 and Jun9-13-9 are noncovalent inhibitors with K_{i} values of 3.96 and 2.10 μM, respectively (Figure S3). The Lineweaver–Burk plots yielded an intercept at the Y-axis, suggesting that both compounds are competitive inhibitors similar to GRL0617 (Figure S3).

**Lead Optimization of SARS-CoV-2 PL^{pro} Inhibitors.** To further optimize the enzymatic inhibition of Jun9-13-7 and Jun9-13-9, 13 structural analogues were purchased from Enamine (Figure 3A), and 34 compounds were synthesized...
Figure 3B to elucidate the structure–activity relationships (SAR). It was found that a hydroxyl substitution on the left phenyl ring is critical for the activity, as methylation led to significant loss of enzymatic inhibition (Jun9-13-9 vs Jun9-25-4). The methyl substitution on the methylene linker is also important for the enzymatic inhibition (Jun9-13-9 vs Jun9-26-4).
Similarly, the ortho-methyl or chloride substitution on the right phenyl ring is critical for the activity (Jun9-13-7 vs Jun9-29-5; Jun9-13-7 vs Jun9-13-4). Next, guided by this initial SAR results, 34 analogues were designed and synthesized (Figure 3B). Nine compounds had IC$_{50}$ values less than 1 μM including Jun9-75-4 (IC$_{50}$ = 0.62 μM), Jun9-85-1 (IC$_{50}$ = 0.66 μM), Jun9-84-3 (IC$_{50}$ = 0.67 μM), Jun9-87-1 (IC$_{50}$ = 0.87 μM), Jun9-72-2 (IC$_{50}$ = 0.67 μM), Jun9-87-2 (IC$_{50}$ = 0.90 μM).
μM), Jun9-75-4 (IC50 = 0.62 μM), Jun9-75-5 (IC50 = 0.56 μM), and Jun9-53-2 (IC50 = 0.89 μM). Among them, Jun9-75-4 was the most potent PLpro inhibitor with an IC50 of 0.62 μM, a 10-fold increase compared to Jun9-13-9 (IC50 = 6.67 μM).
μM). Jun9-75-4 is also 3-fold more potent than GRL0617 (IC50 = 2.05 ± 0.12 μM), representing one of the most potent PLpro inhibitors reported to date.

**Development of FlipGFP Assay for Testing the Cellular Activity of SARS-CoV-2 PLpro Inhibitors.** One of the challenges in SARS-CoV-2 antiviral drug discovery is that SARS-CoV-2 is a biosafety level 3 (BSL-3) pathogen, which limits the number of drug candidates that can be screened. To help prioritize lead compounds for the antiviral assay with infectious SARS-CoV-2, which requires BSL-3 facility, we developed a cell-based FlipGFP assay for SARS-CoV-2 PLpro that is suitable for testing the intracellular activity of PLpro inhibitors in the BSL-2 setting. The two major advantages of a cell-based PLpro assay over the FRET-based enzymatic assay are that (1) it can eliminate compounds that are either cytotoxic or membrane impermeable, and (2) substrate cleavage in the cell cytoplasm recapitulates the physiological process of viral polyprotein cleavage by PLpro in a virus-infected cell. It is known that cysteine proteases are susceptible to redox active compounds as well as nonspecific alkylating chemicals such as ebselen.26,27 The FlipGFP-PLpro assay is expected to rule out such promiscuous compounds since the substrate is cleaved under the reducing intracellular environment.

In the assay design, the 10th and 11th β-strands from the GFP protein were separated from the rest of the GFP β-barrel (β-strands 1–9) (Figure 4A).28−30 The 10th and 11th β-strands were linked through the PLpro cleavage site and a heterodimerized coiled coils E5/K5. In the absence of the PLpro, the 10th and 11th β-strands are restrained and unable to associate with the GFP β-barrel 1–9. When the cleavage site is digested by the PLpro, the 11th β-strand then flips its orientation and associates with GFP β-barrel 1–9 together with the 10th β-strand, leading to restoration of the green fluorescence signal (Figure 4A). A red fluorescent protein mCherry was included within the construct via a “self-cleaving” 2A peptide to act as the transfection control (Figure 4B), and the normalized ratio of green fluorescence signal over red fluorescence signal is proportional to the enzymatic activity of PLpro. Cells transfected with FlipGFP-PLpro but without the PLpro showed no green fluorescence signal (Figure 4C, sixth row), suggesting host proteases are unable to cleave the PLpro substrate sequence, thereby eliminating the background signal interference. Specifically, little or no GFP signal was observed when the cells were transfected with SARS-CoV-2 PLpro and a construct containing either the TEV cleavage site (FlipGFP-TEV) (Figure 4C, fourth row) or the Mpro cleavage site (FlipGFP-Mpro) (Figure 4C, third row). Similarly, little or no GFP signal was observed when the cells were transfected with SARS-CoV-2 Mpro and a construct containing the PLpro cleavage site (FlipGFP-PLpro) (Figure 4C, fifth row). In contrast, strong green fluorescence signals were observed when the cells were transfected with PLpro and FlipGFP-PLpro (Figure 4C, seventh row) or Mpro and FlipGFP-Mpro (Figure 4C, second row).

**Figure 6.** Correlation of the FlipGFP-PLpro, FRET assay results with the antiviral assay results. (A) Correlation of the results between FlipGFP-PLpro assay and the antiviral assay in Vero E6 cells. (B) Correlation of the results between FlipGFP-PLpro assay and the antiviral assay in Caco2-hACE2 cells. (C) Correlation of the results between FRET assay and the antiviral assay in Vero E6 cells. (D) Correlation of the results between FRET assay and the antiviral assay in Caco2-hACE2 cells.
With the established assay condition, we then screened nine most potent PL$^{\text{pro}}$ inhibitors with $IC_{50}$ values less than 1 μM from the FRET-based enzymatic assay (Figure 3). GRL0617 and GC376 were included as positive and negative controls, respectively. Compounds were added 3 h post transfection, and GFP and mCherry fluorescence signals were measured at 48 h post transfection. A dose-dependent decrease of the GFP signal was observed with increasing concentrations of GRL0617 (Figure 4D), and quantification of the normalized GFP/mCherry ratio gave an $EC_{50}$ value of 9.29 ± 3.45 μM. As expected, GC376 had no effect on the intensity of green fluorescence signal ($EC_{50} > 60$ μM) (Figure 4E), suggesting the FlipGFP assay is suitable for the screening of PL$^{\text{pro}}$ inhibitors. Among the nine compounds tested in the cell-based FlipGFP assay, compounds Jun9-53-2, Jun9-72-2, Jun9-75-4, Jun9-85-1, and Jun9-87-1 had EC50 values less than 10 μM, while compounds Jun9-84-3 and Jun9-87-3 were less active with EC50 values of 17.07 μM and 10.16 μM, respectively. Compounds Jun9-75-5 and Jun9-87-2 were not active ($EC_{50} > 50$ μM), despite their potent activity in the FRET-based enzymatic assay (Figure 3 Jun9-75-5, $IC_{50} = 0.56$ μM; Jun9-87-2, $IC_{50} = 0.90$ μM).

**Cellular Antiviral Activity of PL$^{\text{pro}}$ Inhibitors against SARS-CoV-2.** To determine whether there is a correlation between the FlipGFP-PL$^{\text{pro}}$ assay results and the cellular antiviral activity of PL$^{\text{pro}}$ inhibitors, we first tested the nine PL$^{\text{pro}}$ inhibitors selected from the FRET assay with IC50 values less than 1 μM against SARS-CoV-2 (USA-WA1/2020) in Vero E6 cells. GRL0617 inhibited SARS-CoV-2 with an EC50 of 23.64 μM. Compounds Jun9-72-2, Jun9-75-4, Jun9-84-3, Jun9-85-1, and Jun9-87-1 had more potent antiviral activity than GRL0617 with EC50 values of 6.62 μM, 7.88 μM, 8.31 μM, 7.81 μM, and 10.14 μM, respectively (Figure 5A). Compounds Jun9-53-2 and Jun9-87-3 had similar antiviral activity as GRL0617 with EC50 values of 25.19 μM and 22.34 μM, respectively. In contrast, Jun9-75-5 and Jun9-87-2 were not active ($EC_{50} > 60$ μM) (Figure S4).

To further confirm the antiviral activity, we tested the same set of compounds against SARS-CoV-2 in Caco2-hACE2 cells. Caco2-ACE2 expresses TMPRSS2 and is a physiologically relevant cell line for SARS-CoV-2 replication.31−33 Caco2-hACE2 cells were included as positive and negative controls, respectively. Jun9-72-2, Jun9-75-4, Jun9-84-3, Jun9-85-1, and Jun9-87-3 showed improved antiviral activity with EC50 values ranging from 7.90 to 16.22 μM (Figure 5B). Jun9-75-5 and Jun9-87-2 were not active ($EC_{50} > 60$ μM) (Figure S4), which is consistent with the results from the Vero E6 cells.

Overall, three PL$^{\text{pro}}$ inhibitors Jun9-72-2, Jun9-85-1, and Jun9-87-1 were identified as potent SARS-CoV-2 antivirals with EC50 values at or less than 10 μM when tested in both the Vero E6 and Caco2-hACE2 cell lines.

**Correlation between the Results from the FlipGFP PL$^{\text{pro}}$ Assay and the Antiviral Assay.** Plotting the FlipGFP-PL$^{\text{pro}}$ assay results with the antiviral assay results showed that there is a positive correlation in both the Vero E6 and Caco2-hACE2 cell lines with $R^2$ values of 0.86 and 0.89, respectively (Figure 6A,B). Specifically, compounds Jun9-75-5 and Jun9-87-2 with weak activity in the FlipGFP-PL$^{\text{pro}}$ assay ($EC_{50} > 60$ μM and 55.07 μM, Figure 4E) also had no antiviral activity against SARS-CoV-2 (Vero E6 and Caco2-hACE2 cells $EC_{50} > 60$ μM, Figure S4). The remaining seven compounds which had potent activity in the FlipGFP-PL$^{\text{pro}}$ assay also showed potent antiviral activity in both the Vero E6 and Caco2-hACE2 cells (Figure 6A,B). These results suggest that the FlipGFP-PL$^{\text{pro}}$ assay can be used to faithfully predict the cellular antiviral activity of PL$^{\text{pro}}$ inhibitors against infectious SARS-CoV-2. Although the FRET-based enzymatic assay is typically used to select compounds for the antiviral assay, we found there is a poor correlation between the FRET assay results and the cellular antiviral assay results (Figure 6C,D). Taken together, the correlation plots highlighted the advantage of the FlipGFP-PL$^{\text{pro}}$ assay in prioritizing lead compounds for the antiviral assay with infectious SARS-CoV-2.

**X-ray Crystal Structure of SARS-CoV-2 PL$^{\text{pro}}$ in Complex with GRL0617.** The complex structure of SARS-CoV-2 PL$^{\text{pro}}$ with GRL0617 was determined at 2.50 Å resolution, providing insight into its mechanism of inhibition. There are two monomers per asymmetric unit in the P21 space group. Unambiguous electron density reveals that GRL0617 binds to the S3–S4 subpockets of PL$^{\text{pro}}$ (Figure 7A).
complex structure (Figure 7C), demonstrating the nonpolar features of the S4 site as well as the complementarity of the methyl moiety with the core of this subpocket. The amide nitrogen of GRL0617 serves as a hydrogen bond acceptor for the side chain of Asp164, while the amide oxygen accepts a hydrogen bond from the mainchain amide of Gln269. The disubstituted benzene spans the central substrate channel, partially occupying the P5−P3 substrate mainchain binding site, where it forms π−π interactions with the side chains of Tyr268/Gln269, and the backbone amides of Gly163/Asp164. The ortho-methyl group projects toward the catalytic core forming hydrophobic interactions with the S2 site, forcing Leu162 slightly outward compared with the apo structure, while the meta-nitrogen orients toward the S5 site, causing Gln269 to swing inward to accept a hydrogen bond. In parallel to our study, the X-ray crystal structures of SARS-CoV-2 PL^p50 with GRL0617 and its analogues were also released by others with PDB IDs of 7CMD,24 7CJM (C111S), 13 7JIR (C111S, Snyder457), 22 7JIT (C111S, Snyder495), 7JIV (C111S, Snyder530), and 7JIW (C111S, Snyder530). Notably, we are among the first ones to crystallize GRL0617 with the WT SARS-CoV-2 PL^p50.

One of the unique aspects of GRL0617 is that it does not interact with the catalytic core but instead binds to a distal portion of the active site. Other research groups have determined complex structures of PL^p50 with GRL0617 with its catalytic cysteine, Cys 111, mutated to a serine, presumably to increase its propensity to crystallize (PDB ID, 7JIR (2.1 Å) and 7CJM (3.2 Å)).15,22 When the three structures are compared, the GRL0617 adopts a nearly identical pose. Minor
differences in the side chain conformations of Glu 167 and Gln 269 are observed. However, there is a significant difference in the pose of Leu 162 between the WT and the C111S mutants (Figure 7D). In our WT structure, Leu 162 inserts into the core of the protein, where it maintains an interatomic distance of 3.4 Å with the catalytic cysteine. In contrast, Leu 162 of both C111S structures flips outward, toward the solvent. In the higher-resolution structure (PDB ID 7JIR), an acetate from the crystallization condition is modeled in the active site. When superimposed with our WT structure, this acetate clashes with Cys 111 (closest distance 2.5 Å) and Leu 162 (3.0 Å). In the lower-resolution C111S mutant complexed with GRL0617 (PDB ID 7CJM), no acetate is modeled, but Leu 162 adopts the same conformation as the higher-resolution C111S structure (PDB ID 7JIR). Further inspection of the 2Fo − Fc map of 7JCM reveals that there is unmodeled density corresponding to the acetate from PDB ID 7JIR. Interestingly, this experiment did not use acetate in their crystallization condition. Therefore, the density in the catalytic core of both C111S structures likely corresponds to a species of unknown identity that preferentially interacts with a serine residue.

**Molecular Dynamics Simulations of SARS-CoV-2 PLpro with GRL0617, Jun9-53-2, Jun9-72-2, and Jun9-75-4.** The binding interactions between the GRL0617 and the PLpro protein in the X-ray structure with PDB ID 7JRN and the stability of the X-ray structure were further explored using 100 ns MD simulations. The MD simulations show that the complex formed is stable (Figure 8A–C) and did not deviate significantly from the starting crystallographic structure in the protease S4/S3 area, having RMSD values smaller than ca. 2.4 Å for the protein and ca. 2 Å for the ligand (Figure 8C). The MD simulations further verified the stability of the binding interactions inside the broad binding cavity of SARS-CoV-2 PLpro observed in the X-ray structure, as inspected from the MD simulation trajectory and shown in frequency interaction and RMSD plots (Figure 8B). The naphthalene ring of the ligand is positioned in the hydrophobic S4 site, according to the specific binding features of a ISG15 peptidic substrate, e.g., with LXGG sequence (PDB ID 4MOW), 25 where it forms T-shaped π-π stacking with Tyr268 and has dispersion interactions with Pro248, Tyr264, and occasionally with Pro247, while the phenyl ring of the ligand can interact with L162 (Figure 8A,B). Hydrogen bonding interactions stabilize the ligand, e.g., between GRL0617 amide CO and the main chain NH of Gln269; the GRL0617 amide NH and the Asp164 side chain carboxylic acid, and occasionally between the anilino amino group of GRL0617 and Tyr 268 side chain hydroxy group (Figure 8A,B).

Using the structure with PDB ID 7JRN as a template, we docked the potent analogues Jun9-53-2, Jun9-72-2, and Jun9-75-4 in the SARS-CoV-2 PLpro drug-binding site. The stability of the docking poses was explored inside the hydrophobic S4 area using 100 ns MD simulations. The MD simulations show that the complexes formed are stable when the two methyl groups of the CH(CH3)−N(CH3) moiety are in the same side of the space, in a gauche position, in agreement with the observed RMSD values of ligands and protein which are both smaller than ca. 2 Å (Figure 8F,IL).

Compared to GRL0617, in Jun9-53-2, Jun9-72-2, and Jun9-75-4 the carboxamide group has been replaced with a methy lamino group increasing the ligand–receptor hydrogen bonding interaction strength due to the presence of a donor N−H+ group. Thus, in all complexes with Jun-compounds, the N−H+ group is engaged in strong ionic bonding interactions with the side chain carboxylic acid of Asp164 (Figure 8D,G,J,E,H,K) throughout the simulation, shifting the ligands from Q269 toward M208 in the S4 area (Figure 8D,G,J). In this binding orientation, the R166 side chain is moved to stabilize the side chain carboxylic acid anion of D164 with anionic hydrogen bonding (Figure 8D,G,J). All the ligands are stabilized inside the binding area by forming T-shaped π-π stacking between the ligand naphthalene ring and Tyr264 and hydrophobic interactions between naphthalene ring and Pro248, Pro247 (Figure 8D,G,J,E,H,K). In the ligand Jun9-75-4 the indole ring NH forms hydrogen bonding interactions with Gln269, while in ligand Jun9-72-2 the donor phenol hydroyxyl group forms a hydrogen bond with the main chain NH group of L162, which is occasionally bridged with a water molecule (Figure 8D,G,J,E,H,K).

**CONCLUSION**

Given the tremendous impact of the COVID-19 pandemic, the SARS-CoV outbreak in 2003 was a dire warning that was gravely overlooked in retrospect. Looking forward, it is imperative that therapeutics are developed that are not only effective against SARS-CoV-2 but against future strains of similar coronaviruses. PLpro is a high-profile drug target, partially because it is highly conserved between SARS-CoV and SARS-CoV-2, sharing 83% sequence similarity. Inhibitors like GRL0617 are equally effective against both viruses, with a K_i of 0.49 μM and 0.57 μM, against SARS-CoV PLpro and SARS-CoV-2 PLpro. 23 Likewise, all critical active site residues that interact with GRL0617 are conserved. Consequently, the binding poses are nearly identical (Figure S5). These similarities would indicate that PLpro inhibitors might retain their activity against beta coronaviruses that might emerge in the future.

Previous attempts to discover SARS-CoV-2 PLpro inhibitors through HTS have failed to identify hits with improved enzymatic inhibition and cellular antiviral activity. 12,13 Structural analogues of GRL0617 were also designed and synthesized; however, none showed improved enzymatic inhibition. 22 Part of the reason for the difficulty in targeting SARS-CoV-2 PLpro is the lack of S1 and S2 pockets, which leaves only S3 and S4 pockets for inhibitor binding. The majority of the cysteine protease inhibitors are covalent inhibitors targeting the catalytic cysteine, 12 and it remains a challenge to develop noncovalent cysteine protease inhibitors with a similar potency as the covalent inhibitors. Among the reported SARS-CoV or SARS-CoV-2 PLpro inhibitors, GRL0617 is one of the most potent compounds. However, it had weak antiviral activity (Vero E6: EC_50 = 23.64 μM; Caco2-hACE2: EC_50 = 19.96 μM). In this study, we aim to identify more potent SARS-CoV-2 PLpro inhibitors through a HTS. On the basis of two promising hits Jun9-13-7 and Jun9-13-9, a library of analogues was designed and synthesized, among which several compounds had sub-micromolar IC_50 values in the FRET-based enzymatic assay. To alleviate the burden of relying on BSL-3 facility to test the antiviral activity of PLpro inhibitors, we developed the cell-based FlipGFP-PLpro assay, which can be used to quantify the intracellular enzymatic inhibition of PLpro in a BSL-2 lab. The FlipGFP-PLpro assay is a close mimic of the virus-infected cell in which PLpro cleaves its substrate in the native intracellular reducing environment. The advantage of the FlipGFP-PLpro assay over the standard FRET-based enzymatic assay is that it can rule out compounds
that are either cytotoxic or membrane impermeable or nonspecifically modifying the catalytic cysteine through oxidation or alkylation. Our results showed there is a positive correlation between the results of FlipGFP-PLpro assay and the antiviral assay in both the Vero E6 and Caco2-hACE2 cells. In contrast, the correlation between the FRET assay results and the antiviral assay results is poor. The FlipGFP-PLpro assay can be performed in the BSL-2 setting, which alleviates the resources and financial burdens associated with screening a large number of compounds in the BSL-3 facility. This is expected to speed up the drug discovery process. In total, three PLpro inhibitors Jun9-72-2, Jun9-85-1, and Jun9-87-1 were identified as potent SARS-CoV-2 antivirals with EC_{50} values at or less than 10 μM when tested in both the Vero E6 and Caco2-hACE2 cell lines.

We also solved the X-ray crystal structure of the wild-type SARS-CoV-2 PLpro in complex with GRL0617. To SARS-CoV-2 induced a conformational change in the BL2 loop to the more closed conformation. In contrast, a larger inhibitor VIR251 stabilizes the BL2 loop in the open conformation. The intrinsic flexibility of the BL2 loop implies that structurally diverse inhibitors might be able to fit in the S3–S4 pockets.

As shown by the MD simulations, the replacement of the carboxamide group in GRL0617 to the trialkyl ammonium in Jun9-53-2, Jun9-72-2, and Jun9-75-4 affects the binding interactions inside the receptor-binding region. In comparison to GRL0617, the N−H group in Jun9-53-2, Jun9-72-2, and Jun9-75-4 is engaged in strong ionic hydrogen bonding interactions with a side chain of Asp164, participating in another stabilizing ionic hydrogen bonding interactions with Arg166, which pulls the ligands inside the receptor-binding region from the hydrogen-bonded Gln269 to a new T-shaped π−π stacking with the Ty264 instead of Tyr268 in GRL0617. All the four ligands form hydrophobic interactions between naphthalene ring and Pro248. Overall, these features might explain the higher potency of Jun9-53-2, Jun9-72-2, and Jun9-75-4 compared to GRL0617.

In conclusion, the SARS-CoV-2 PLpro inhibitors discovered in this study represent promising hits for further development as SARS-CoV-2 antivirals, the FlipGFP-PLpro assay is a suitable surrogate for testing the cellular activity of PLpro inhibitors in the BSL-2 setting, and the results can be used to help prioritize leads for the antiviral assay.

**Materials and Methods**

**Cell Lines and Viruses.** VERO E6 cells (ATCC, CRL-1586) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 5% heat-inactivated FBS in a 37 °C incubator with 5% CO_{2}. Caco2 cells expressing human ACE2 (Caco2-hACE2) were established by transducing Caco2 cells (ATCC HTB-37) with lentiviral particles derived with pWPI-IRES-Puro-Ak-ACE2 (a gift from Sonja Best; Addgene plasmid #154985).

SARS-CoV-2, isolate USA-WA1/2020 (NR-2281), was obtained through BEI Resources and propagated once on VERO E6 cells before it was used for this study. Studies involving the SARS-CoV-2 were performed at the UTHSCSA biosafety level-3 laboratory by personnel wearing powered air-purifying respirators.

**Protein Expression and Purification.** Detailed expression and purification of C-terminal His tagged SARS-CoV-2 PLpro (PLpro-His) were described in our previous publication. Briefly, the SARS-CoV-2 papain-like protease (PLpro) gene (ORF 1ab 1564–1876) from strain BetaCoV/Wuhan/ WIV04/2019 with E. coli codon optimization in the pET28b(-) vector was ordered from GenScript. The pET28b(-) plasmid was transformed into BL21 (DE3) cells, and protein expression was induced with 0.5 mM IPTG when the OD_{600} was around 0.8 for 24 h at 18 °C. Then cells were harvested and lysed, the PLpro-His protein was purified with a single Ni−NTA resin column, and eluted PLpro-His was dialyzed against a 100-fold volume dialysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM DTT) in a 10 000 kDa molecular weight cutoff dialysis tubing.

The expression and purification of untagged SARS-CoV-2 PLpro (PLpro) were carried out as follows: the SARS-CoV-2 PLpro gene (ORF 1ab 1564–1876) was subcloned from the pET28b(+) to pe-SUMO vector according to the manufacturer’s protocol (LifeSensors Inc., Malvern, PA). The forward primer with the Bsa I site is GCGGTCTCAGGTGTAAGTTCGACCATCAAGTTTTTACACATGTTCTAGATTACT-TGATGTTGGTTGTTGAGCTGTTCTC. SUMO-tagged protein was expressed and purified as PLpro-His protein. The SUMO tag was removed by incubation with SUMO protease 1 at 4 °C overnight, and the free SUMO tag was removed by application of another round of Ni-NTA resin. The purity of the protein was confirmed with a SDS-PAGE gel.

The expression and purification of SARS-CoV-2 Mpro with unmodified N- and C-termini were reported in previous studies.

**Peptide Synthesis.** The SARS-CoV-2 PLpro FRET substrate Dabcyl-FTLRGG/APTKV(Edans) and the SARS-CoV-2 Mpro FRET substrate Dabcyl-FTLRGG/APTKV(Edans) were synthesized by solid-phase synthesis through iterative cycles of coupling and deprotection using the previously optimized procedure.

Ub-AMC and ISG15-AMC were purchased from Boston Biochem (catalog no. U-550-050 and UL-553-050, respectively).

**Compound Synthesis and Characterization.** Details for the synthesis procedure and characterization for compounds can be found in the Supporting Information.

**Enzymatic Assays.** The high-throughput screening was carried out in 384-well format. One microliter of 2 mM library compound was added to 50 μL of 200 nM PLpro-His protein in a PLpro reaction buffer (50 mM HEPES pH 7.5, 5 mM DTT and 0.01% Triton X-100) and was incubated at 30 °C for 1 h. The reaction was initiated by adding 1 μL of 1 mM PLpro FRET substrate. The end-point fluorescence signal was measured after 3 h incubation at 30 °C with a Cytagen 5 image reader with filters for excitation at 360/40 nm and emission at 460/40 nm. The final testing compound concentration is ~40 μM, and the FRET substrate concentration is ~20 μM; a control plate as in Figure 1 was included in every batch of screening.

The diversity compound library consisting of 50,240 compounds was purchased from Enamine (catalog no. 781270).

For the measurements of Keq/Vmax with Peptide-Edans as a substrate, the final PLpro protein concentration is 200 nM, and the substrate concentration ranges from 0 to 200 μM; with Ub-AMC as a substrate, the final PLpro protein concentration is 50 nM, and the Ub-AMC concentration ranges from 0 to 40 μM; with ISG15-AMC as a substrate, the final PLpro protein
concentration is 2 nM, and the ISG15-AMC concentration ranges from 0 to 15 μM. The reaction was monitored in a Cytation 5 image reader with filters for excitation at 360/40 nm and emission at 460/40 nm at 30 °C for 1 h. The initial velocity of the enzymatic reaction was calculated from the initial 10 min enzymatic reaction and was plotted against the substrate concentrations in Prism 8 with a Michaelis–Menten function.

For the IC₅₀ measurement with FRET peptide-Edans substrate: the reaction was carried out in 96-well format with 200 nM PLpro protein as described previously. For the IC₅₀ measurements with Ub-AMC or ISG15-AMC substrate, the reaction was carried out in 384-well format. The final PLpro protein concentration is 50 nM, and substrate concentration is 2.5 μM when Ub-AMC is applied; the final PLpro protein concentration is 2 nM, and substrate concentration is 0.5 μM when ISG15-AMC is applied.

For the Lineweaver–Burk plots of GRL0617, Jun9-13-7, and Jun9-13-9, the assay was carried as follows: 50 μL of 400 nM PLpro protein was added to 50 μL of reaction buffer containing testing compound and various concentrations of FRET peptide-Edans substrate to initiate the enzyme reaction. The initial velocity of the enzymatic reaction with and without testing compounds was calculated by linear regression for the first 10 min of the kinetic progress curve, and then plotted against substrate concentrations in Prism 8 with a Michaelis–Menten equation and linear regression of double reciprocal plot.

The main protease (Mpro) enzymatic assays were carried out in Mpro reaction buffer containing 20 mM HEPES pH 6.5, 120 mM NaCl, 0.4 mM EDTA, 20% glycerol, and 4 mM DTT as described previously. A total of 50 ng of pcDNA3-TVE-flipGFP-T2A-mCherry plasmid was used in Addgene (catalog no. 124429). SARS-CoV-2 PLpro cleavage site LRGGAPT K or SARS-CoV-2 Mpro cleavage site AVLQSGFR was introduced into pcDNA3-FlipGFP-T2A-mCherry via overlapping PCRs to generate a fragment with Scal and HindIII sites at the ends. SARS-CoV-2 Mpro and PLpro expression plasmids pcDNA3.1 SARS2Mpro and pcDNA3.1 SARS2 PLpro were ordered from Genscript (Piscataway NJ) with codon optimization.

For transfection, 96-well Greiner plate (catalog no. 655090) was seeded with 293T cells to overnight 70–90% confluency. A total of 50 ng of pcDNA3-flipGFP-T2A-mCherry plasmid and 50 ng of protease expression plasmid pcDNA3.1 were used each well in the presence of transfection reagent TransIT-293 (Mirus). Three hours after transfection, 1 μL of testing compound was added to each well at 100-fold dilution. Images were acquired 2 days after transfection with a Cytation 5 imaging reader (Biotek) GFP and mCherry channels and were acquired 2 days after transfection with a Cytation 5 image reader (Biotek). The total number of cells, as indicated by the nuclei staining, and the fraction of the infected cells, as indicated by the NP staining, were quantified by the cellular analysis module of the Gen5 software (BioTek).

Crystallographic and Structure Determination. SARS-CoV-2 PLpro-His (P1-pro-His) protein was concentrated and loaded to a HiLoad 16/60 Superdex 75 size exclusion column (GE Healthcare) pre-equilibrated with 20 mM Tris pH 8.0 and 5 mM NaCl. Peak fractions were pooled and incubated with GRL0617 in a 1:1 molar ratio for 1 h at room temperature and then concentrated to 8 mg/mL. PLpro crystals were grown in a hanging-drop, vapor-diffusion apparatus by mixing 0.75 μL of 8 mg/mL PLpro-GRL0617 with 0.75 μL of well solution (30% PEG 4000, 0.2 M Li2SO4 and 0.1 M Tris pH 8.5). Crystals were transferred to a cryoprotectant solution containing 30% PEG 4000, 0.2 M Li2SO4, 0.1 M Tris pH 8.5, and 15% glycerol, before being flash frozen in liquid nitrogen. X-ray diffraction data for SARS-CoV-2 PLpro + GRL0617 was collected on the SBC 19-BM beamline at the Advanced Photon Source (APS) in Argonne, IL, and processed with the HKL2000 software suite. The CCP4 versions of MOLREP
were used for molecular replacement using a previously solved apo SARS-CoV-2 PL<sup>pro</sup> structure, PDB ID: 6WZU as a reference model. Rigid and restrained refinements were performed using REFMAC, and model building was performed with COOT. Protein structure figures were made using PyMOL (Schrödinger, LLC).

**MD Simulations.** MD simulations were carried out to the bound GRL0617, Jun9-53-2, Jun9-72-2, and Jun9-75-4 with PL<sup>pro</sup> prepared as described previously from the experimental structure of SARS-CoV-2 PL<sup>pro</sup> with GRL0617 (PDB ID 2JRN). Each complex was solvated using the TIP3P water model. Using the “System Builder” utility of Schrödinger Desmond v.11.1, each complex was embedded in an orthorhombic water box extending beyond the solute 10 Å in the x,y,z direction leading to 14 500 waters. Na<sup>+</sup> and Cl<sup>-</sup> ions were placed in the water phase to neutralize the systems and to reach the experimental salt concentration of 0.15 M NaCl. The total number of atoms was ca. 48 000.

The OPLS-2005 force field was used to model all protein and ligand interactions and lipids. The particle mesh Ewald method (PME) was employed to calculate long-range electrostatic interactions with a grid spacing of 0.8 Å. van der Waals and short-range electrostatic interactions were smoothly truncated at 9.0 Å. The Langevin thermostat was utilized to maintain a constant temperature in all simulations, and the Berendsen barostat was used to control the pressure. Periodic boundary conditions were applied (73 × 102 × 65) Å<sup>3</sup>. The equations of motion were integrated using the multistep RESPMA integrator with an inner time step of 2 fs for bonded interactions and nonbonded interactions within a cutoff of 9 Å. An outer time step of 6.0 fs was used for nonbonded interactions beyond the cutoff. Each system was equilibrated in MD simulations with a default protocol for water-soluble proteins provided in Desmond, which consists of a series of restrained MD simulations designed to relax the system while not deviating substantially from the initial coordinates.

The first simulation was a Brownian dynamics run for 100 ps at a temperature of 10 K in the NVT (constant number of particles, volume, and temperature) ensemble with solute heavy atoms restrained with a force constant of 50 kcal mol Å<sup>-2</sup>. The Langevin thermostat was applied in the NVT ensemble and a MD simulation for 12 ps with solute heavy atoms restrained with a force constant of 50 kcal mol Å<sup>-2</sup>. The velocities were randomized, and MD simulation for 12 ps was performed in the NPT (constant number of particles, pressure, and temperature) ensemble and a Berendsen barostat with solute heavy atoms equally restrained at 10 K and another one at 300 K. The velocities were again randomized, and unrestrained MD simulation for 24 ps was performed in the NPT ensemble. The above-mentioned equilibration was followed by 100 ns simulation without restraints. Two simulations were performed in a workstation with GTX 970. The visualization of the produced trajectories and structures was performed using Maestro or programs Chimera and VMD.

**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.1c00519.

**Accession Codes** The drug-bound complex structures for SARS-CoV-2 PL<sup>pro</sup> with GRL0617 were deposited in the Protein Data Bank with accession numbers of 7JRN.

**AUTHOR INFORMATION**

**Corresponding Authors**

Jun Wang — Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, Tucson, Arizona 85721, United States; orcid.org/0000-0002-4845-4621; Phone: 520-626-1366; Email: junwang@pharmacy.arizona.edu; Fax: 520-626-0749

Yu Chen — Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, Florida 33612, United States; Phone: 813-974-7809; Email: ychen1@usf.edu

**Authors**

Chunlong Ma — Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, Tucson, Arizona 85721, United States

Michael Dominic Sacco — Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, Florida 33612, United States

Zilei Xia — Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, Tucson, Arizona 85721, United States

George Lambrinidis — Section of Pharmaceutical Chemistry, Department of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, 15771 Athens, Greece; orcid.org/0000-0002-2820-9338

Julia Alma Townsend — Department of Chemistry and Biochemistry, The University of Arizona, Tucson, Arizona 85721, United States

Yanmei Hu — Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, Tucson, Arizona 85721, United States

Xiangzi Meng — Department of Microbiology, Immunology and Molecular Genetics, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229, United States

Tommy Szeto — Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, Tucson, Arizona 85721, United States

Mandy Ba — Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, Tucson, Arizona 85721, United States

Xiujuan Zhang — Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, Florida 33612, United States

Maura Gongora — Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, Florida 33612, United States

Fushun Zhang — Department of Microbiology, Immunology and Molecular Genetics, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229, United States

Michael Thomas Marty — Department of Chemistry and Biochemistry, The University of Arizona, Tucson, Arizona 85721, United States
Yan Xiang — Department of Microbiology, Immunology and Molecular Genetics, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229, United States
Antonios Kolocouris — Section of Pharmaceutical Chemistry, Department of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, 15771 Athens, Greece; orcid: 0000-0001-6110-1903

Complete contact information is available at: https://pubs.acs.org/10.1021/acscentsci.1c00519

Author Contributions
#C.M., M.D.S., and Z.X. contributed equally to this work. J.W. and C.M. conceived and designed the study; Z.X. designed and synthesized the PLRe inhibitors; C.M. expressed the PLRe with the assistance of T.S.; C.M. performed the HTS, IC50 determination, thermal shift binding assay, and enzymatic kinetic studies with the assistance of M.B.; C.M. developed the FlipGFP-PLRe assay. M.D.S. carried out MRe crystallization and structure determination with the assistance of X.Z. and M.G. and analyzed the data with Y.C.; J.T. performed the kinetic studies with the assistance of Y.X.; G.L. and A.K. performed the MD simulations; J.W. and Y.C. established CaCO2-hACE2 cell line and performed the SARS-native mass spectrometry experiments with guidance from M.G. and A.K. performed the MD simulations; J.W. and Y.C. secured funding and supervised the study; J.W., Y.C., and M.S. wrote the manuscript with the input from the others.

Notes
The authors declare the following competing financial interest(s): A patent was filed to claim the compounds described herein as potential SARS-CoV-2 antivirals.

ACKNOWLEDGMENTS
This research was partially supported by the National Institutes of Health (NIH) (Grants AI147325 and AI147046) and the Arizona Biomedical Research Centre Young Investigator grant (ADHS18-198859) to J.W. J.A.T. and M.T.M. were funded by the National Institute of General Medical Sciences and National Institutes of Health (Grant R35 GM128624 to M.T.M.). Y.H. and J.A.T. were supported by T32 GM008804. We thank Michael Kemp for assistance with crystallization and X-ray diffraction data collection. We also thank the staff members of the Advanced Photon Source of Argonne National Laboratory, particularly those at the Structural Biology Center (SBC), with X-ray diffraction data collection. SBC-CAT is operated by UChicago Argonne, LLC, for the U.S. Department of Energy, Office of Biological and Environmental Research under contract DE-AC02-06CH11357. The SARS-CoV-2 experiments were supported by a COVID-19 pilot grant from UTHSCSA and NIH Grant AI15638 to Y.X. SARS-related coronavirus 2, isolate USA-WA1/2020 (NR-52281) was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH.

REFERENCES
(1) Hu, B.; Guo, H.; Zhou, P.; Shi, Z. L. Characteristics of SARS-CoV-2 and COVID-19. Nat. Rev. Microbiol. 2021, 19 (3), 141–154.
(2) Piroth, L.; Cottenet, J.; Mariet, A.-S.; Bonniard, P.; Blot, M.; Tubert-Bitter, P.; Quantin, C. Comparison of the characteristics, morbidity, and mortality of COVID-19 and seasonal influenza: a nationwide, population-based retrospective cohort study. Lancet Respir. Med. 2021, 9 (3), 251–259.
(3) Bar-On, Y. M.; Flamholz, A.; Phillips, R., Milo, R. SARS-CoV-2 (COVID-19) by the numbers. Cell 2020, 9, e57309.
(4) Li, Y.; Tanchov, R.; Smaut, J.; Liu, C.; Watkins, S.; Zhou, Q. A Comprehensive Review of the Global Efforts on COVID-19 Vaccine Development. ACS Cent. Sci. 2021, 7 (4), S12–S33.
(5) Eastman, R. T.; Roth, J. S.; Brimacombe, K. R.; Simeonov, A.; Shen, M.; Patnaik, S.; Hall, M. D. Remdesivir: A Review of Its Discovery and Development Leading to Emergency Use Authorization for Treatment of COVID-19. ACS Cent. Sci. 2020, 6 (5), 672–683.
(6) Lauring, A. S.; Hodcroft, E. B. Genetic Variants of SARS-CoV-2—What Do They Mean? JAMA 2021, 325 (6), 529–531.
(7) Ma, C.; Sacco, M. D.; Hurst, B.; Townsend, J. A.; Hu, Y.; Szeto, T.; Zhang, X.; Tarbet, B.; Marty, M. T.; Chen, Y.; Wang, J. Boceprevir, GC-376, and calpain inhibitors II, XII inhibit SARS-CoV-2 viral replication by targeting the viral main protease. Cell Res. 2020, 30 (8), 678–692.
(8) Sacco, M. D.; Ma, C.; Lagarias, P.; Gao, A.; Townsend, J. A.; Meng, X.; Dube, P.; Zhang, X.; Hu, Y.; Kitamura, N.; Hurst, B.; Tarbet, B.; Marty, M. T.; Kolocouris, A.; Xiang, Y.; Chen, Y.; Wang, J. Structure and inhibition of the SARS-CoV-2 main protease reveal strategy for developing dual inhibitors against M(pro) and capsid NS2. J. Virol. Adv. 2020, 6 (50), eabe0751.
(9) Rut, W.; Lv, Z.; Zmudzinski, M.; Patchett, S.; Nakay; D.; Snips, S. J.; El Oualif, F.; Huang, T. T.; Bekes, M.; Drag, M.; Olsen, S. K. Activity profiling and crystal structures of inhibitor-bound SARS-CoV-2 papain-like protease: A framework for anti-COVID-19 drug design. Sci. Adv. 2020, 6, eabd4596.
(10) Freitas, B. T.; Durie, I. A.; Murray, J.; Longo, J. E.; Miller, H. C.; Crich, D.; Hogan, R. J.; Tripp, R. A.; Pegan, S. D. Characterization and Nonspecific Inhibition of the Deubiquitinase and deISGylase Activity of SARS-CoV-2 Papain-Like Protease. ACS Infect. Dis. 2020, 6 (8), 2099–2109.
(11) Shin, D.; Mukherjee, R.; Grewe, D.; Bojkova, D.; Baek, K.; Bhattacharya, A.; Schulz, L.; Widera, M.; Meldhipour, A. R.; Tascher, G.; Geurink, P. P.; Wilhelm, A.; van der Heden van Noort, G. J.; Ovaa, H.; Müller, S.; Knobeloch, K.-P.; Rajalingam, K.; Schulman, B. A.; Cinati, J.; Hummer, G.; Ciesek, S.; Dííl, I. Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity. Nature 2020, 587 (7835), 657–662.
(12) Klemm, T.; Ebert, G.; Calleja, D. J.; Allison, C. C.; Richardson, L. W.; Bernardino, J. P.; Lu, B. G.; Kuchel, N. W.; Gromhahn, C.; Shibata, Y.; Gan, Z. Y.; Cooney, J. P.; Doerflinger, M.; Au, A. E.; Blackmore, T. R.; van der Heden van Noort, G. J.; Geurink, P. P.; Ovaa, H.; Newman, J.; Riboldi-Tunnicliffe, A.; Czabotar, P. E.; Mitchell, J. P.; Feltham, R.; Lechtenberg, B. C.; Lowes, K. N.; Dewson, G.; Pellegrini, M.; Lessene, G.; Komander, D. Mechanism and inhibition of the papain-like protease. PLpro, of SARS-CoV-2. EMBO J. 2020, 39 (18), e106275.
(13) Fu, Z.; Huang, B.; Tang, J.; Liu, S.; Liu, M.; Ye, Y.; Liu, Z.; Xiong, Y.; Zhu, W.; Cao, D.; Li, J.; Niu, X.; Zhou, H.; Zhao, Y. J.; Zhang, G.; Huang, H. The complex structure of SARS-CoV-2 PLpro reveals a hot spot for antiviral drug discovery. Nature Commun. 2021, 12 (1), 488.
(14) Berlin, D. A.; Gulick, R. M.; Martinez, F. J. Severe Covid-19. N. Engl. J. Med. 2020, 383 (25), 2451–2460.
(15) Kalil, A. C.; Patterson, T. F.; Mehta, A. K.; Tomashek, K. M.; Wolfe, C. R.; Ghazaryan, V.; Marconi, V. C.; Ruiz-Palacios, G. M.; Hsieh, L.; Kline, S.; Tapson, V.; Ivone, N. M.; Jain, M. K.; Sweeney, D. A.; El Sahly, H. M.; Branche, A. R.; Regalado Pineda, J. J.; Lye, D. C.; Sandkovsky, U.; Luektayemeyer, A.; Cohen, S. H.; Finberg, R. W.; Jackson, P. E. H.; Taiwo, B.; Paules, C. I.; Arguinchona, H.; Erdmann, N.; Ahuja, N.; Frank, M.; Oh, M.-d.; Kim, E.-s.; Tan, S. Y.; Mularksi, R. A.; Nielsen, H.; Ponce, P. O.; Taylor, B. S.; Larson, L.; Rouphael, N. G.; Saklawi, Y.; Cantos, V. D.; Ko, E. R.; Engemann, J. J.; Amin, A. N.; Watanabe, M.; Billings, J.; Elie, M.-C.; Davey, R. T.; Burgess, T. H.; Ferreira, J.; Green, M.; Makowski, M.; Cardoso, A.; de Bono, S.; Bonnett, T.; Proschan, M.; Deye, G. A.; Dempsey, N.; Nayak, S. U.;...

https://doi.org/10.1021/acscentsci.1c00519
Dodd, L. E.; Beigel, J. H. Baricitinib plus Remdesivir for Hospitalized Adults with Covid-19. N. Engl. J. Med. 2021, 384 (8), 795–807.

(17) The RECOVERY Collaborative Group. N. Engl. J. Med. 2020, 384 (8), 693–704, DOI: 10.1056/NEJMoa2021436.

(18) Zhang, L. L.; Lin, D. Z.; Sun, X. Y. Y.; Curth, U.; Drosten, C.; Sauererling, L.; Becker, S.; Rox, K.; Higenfeld, R. Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved alpha-ketoamide inhibitors. Science 2020, 368 (6489), 409–412.

(19) Dai, W.; Zhang, B.; Jiang, X. M.; Su, H.; Li, J.; Zhao, Y.; Xie, X.; Jin, Z.; Peng, J.; Liu, F.; Li, C.; Li, Y.; Bai, F.; Wang, H.; Cheng, X.; Cen, X.; Hu, S.; Yang, X.; Wang, J.; Liu, X.; Xiao, G.; Jiang, H.; Rao, Z.; Zhang, L. K.; Xu, Y.; Yang, H.; Liu, H. Structure-based design of antiviral drug candidates targeting the SARS-CoV-2 main protease. Science 2020, 368 (6497), 1331–1335.

(20) Qiao, J.; Li, Y. S.; Zeng, R.; Liu, F. L.; Luo, R. H.; Huang, C.; Wang, Y. F.; Zhang, J.; Quan, B.; Shen, C.; Mao, X.; Liu, X.; Sun, W.; Yang, W.; Ni, X.; Wang, K.; Xu, L.; Duan, Z. L.; Zou, Q. C.; Zhang, H. L.; Qu, W.; Long, Y. H.; Li, M. H.; Yang, R. C.; Liu, X.; You, J.; Zhou, Y.; Yao, R.; Li, W. P.; Liu, J. M.; Chen, P.; Liu, Y.; Lin, G. F.; Yang, X.; Zou, J.; Li, L.; Hu, Y.; Lu, G.-W.; Li, W.-M.; Wei, Y.-Q.; Zheng, Y.-T.; Lei, J.; Yang, S. SARS-CoV-2 Mpro inhibitors with antiviral activity in a transgenic mouse model. Science 2021, 371, 1374–1378.

(21) Boras, B.; Jones, R. M.; Anson, B. J.; Arendsen, D.; Aschenbrenner, L.; Bakowski, M. A.; Beutler, N.; Binder, J.; Chen, E.; Eng, H.; Hammond, J.; Hoffman, R.; Kadar, E. P.; Kania, R.; Kimoto, E.; Kirkpatrick, M. G.; Lanyon, L.; Lendy, E. K.; Lillis, J. R.; Joachimiak, A. Structure of papain-like protease from SARS-CoV-2 resolved by X-ray crystallography. Science 2020, 369 (6507), 1331–1335.

(22) Osiptuk, J.; Azizi, S.-A.; Dvorkin, S.; Endres, M.; Jadhav, S.; Kader, E. P.; Kania, R.; Sathish, J. G.; Steppan, C.; Ticehurst, M.; Connor, R.; Ogilvie, K.; Owen, D.; Pettersson, M.; Reese, M. R.; Schughart, K.; Pohlmann, S. TMPRSS2 and TMPRSS4 facilitate SARS-CoV-2 entry into human cells. J. Virol. 2020, 94 (22), e202442118.

(23) Barretto, N.; Jukneliene, D.; Ratia, K.; Pegan, S.; Takayama, J.; Sleeman, K.; Coughlin, S. R.; Shu, X. Designing a Green Fluorogenic Protease Reporter by Flipping a Beta Strand of GFP for Imaging Apoptosis in Mice. J. Am. Chem. Soc. 2019, 141 (11), 4526–4530.

(24) Hoffmann, M.; Kleine-Weber, H.; Schroeder, S.; Kruger, N.; Herrler, T.; Erichsen, S.; Schiersgen, T. S.; Herrler, G.; Wu, N. H.; Nitsche, A.; Muller, M. A.; Drosten, C.; Pohlmann, S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell 2020, 181 (2), 271–280.

(25) Bertram, S.; Glowacka, I.; Blazewejksa, P.; Solleux, E.; Allen, P.; Danisch, S.; Steffen, I.; Choi, S. Y.; Park, Y.; Schneider, H.; Schughart, K.; Pohlmann, S. TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. J. Virol. 2010, 84 (19), 10016–25.

(26) Filipacz, J. M.; Lee, S.; Wu, C.-T.; Garcia-Knight, M.; Yang, J.; Nakayama, T.; Nayak, J. V.; Jackson, P. K.; Andino, R.; Shu, X. Ethacridine inhibits SARS-CoV-2 by inactivating viral particles in cellular models. bioRxiv 2020, 2020.10.28.359042.

(27) Zhang, Q.; Scheips, A.; Huang, H.; Yang, J.; Ma, W.; Torza, J.; Zhang, S. Q.; Yang, L.; Wu, H.; Nonell, S.; Dong, Z.; Kornberg, T. B.; Coughlin, S. R.; Shu, X. Designing a Green Fluorogenic Protease Reporter by Flipping a Beta Strand of GFP for Imaging Apoptosis in Animals. J. Am. Chem. Soc. 2019, 141 (11), 4526–4530.

(28) Murshudov, G. N.; Skubák, P.; Lebedev, A. A.; Pannu, N. S.; Steiner, R. A.; Nicholls, R. A.; Winn, M. D.; Long, F.; Vagin, A. A. MOLREP. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2004, 60 (12), 2126–2132.

(29) Moshaverinia, M.; Amin, M.; Ghaemmaghami, S.; Behzadi, N.; Arefi, M.; Asef, R.; Shojaie, S. Inactivating viral proteins via comparison with accurate quantum chemical calculations as therapeutic targets: does selectivity matter? A systematic review of Mpro inhibitors. Acta Pharmacol. Transl Sci. 2020, 3 (6), 1265–1277.
Darden, T.; York, D.; Pedersen, L. Particle mesh Ewald: An \(N\)-\(\log(N)\) method for Ewald sums in large systems. *J. Chem. Phys.* 1993, 98 (12), 10089–10092.

Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen, L. G. A Smooth Particle Mesh Ewald Method. *J. Chem. Phys.* 1995, 103 (19), 8577–8593.

Izaguirre, J. A.; Catarello, D. P.; Wozniak, J. M.; Skeel, R. D. Langevin stabilization of molecular dynamics. *J. Chem. Phys.* 2001, 114 (5), 2090–2098.

Feller, S. E.; Zhang, Y. H.; Pastor, R. W.; Brooks, B. R. Constant-Pressure Molecular-Dynamics Simulation - the Langevin Piston Method. *J. Chem. Phys.* 1995, 103 (11), 4613–4621.

Humphrey, D. D.; Friesner, R. A.; Berne, B. J. A Multiple-Time-Step Molecular-Dynamics Algorithm for Macromolecules. *J. Phys. Chem.* 1994, 98 (27), 6885–6892.

Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 2004, 25 (13), 1605–12.