Discovery of Di- and Trihaloacetamides as Covalent SARS-CoV-2
Main Protease Inhibitors with High Target Specificity

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ABSTRACT: The main protease (Mpro) is a validated antiviral drug target of SARS-CoV-2. A number of Mpro inhibitors have now advanced to animal model study and human clinical trials. However, one issue yet to be addressed is the target selectivity over host proteases such as cathepsin L. In this study we describe the rational design of covalent SARS-CoV-2 Mpro inhibitors with novel cysteine reactive warheads including dichloroacetamide, dibromoacetamide, tribromoacetamide, 2-bromo-2,2-dichloroacetamide, and 2-chloro-2,2-dibromoacetamide. The promising lead candidates Jun9-62-2R (dichloroacetamide) and Jun9-88-6R (tribromoacetamide) had not only potent enzymatic inhibition and antiviral activity but also significantly improved target specificity over caplain and cathepsins. Compared to GC-376, these new compounds did not inhibit the host cysteine proteases including calpain I, cathepsin B, cathepsin K, cathepsin L, and caspase-3. To the best of our knowledge, they are among the most selective covalent Mpro inhibitors reported thus far. The cocrystal structures of SARS-CoV-2 Mpro with Jun9-62-2R and Jun9-57-3R reaffirmed our design hypothesis, showing that both compounds form a covalent adduct with the catalytic C145. Overall, these novel compounds represent valuable chemical probes for target validation and drug candidates for further development as SARS-CoV-2 antivirals.

In combating the COVID-19 pandemic, researchers from different disciplines work relentlessly to discover countermeasures. Drug repurposing led to the identification of remdesivir as the first FDA-approved SARS-CoV-2 antiviral. EIDD-2801, another viral polymerase inhibitor discovered through a similar approach, is in human clinical II/III trials. Among the drug targets exploited, viral polymerases including the main protease (Mpro) and the papain-like protease (PLpro) are the most extensively studied. The Mpro is a cysteine
concern for some of the most advanced Mpro inhibitors an analogue of GC-376 of target specificity has yet received FDA approval, and the lack catastrophic failures in the later clinical studies. Cysteine essential to address the target selectivity issue early on to avoid the selectivity profiling has thus far been largely neglected. It is essential to address the target selectivity issue early on to avoid catastrophic failures in the later clinical studies. Cysteine protease inhibitor has yet received FDA approval, and the lack of target specificity might be the culprit.

The majority of current reported SARS-CoV-2 Mpro inhibitors are peptidomimetic covalent inhibitors with a reactive warhead such as ketone, aldehyde, or ketoamide. Some of the promising examples include the Pfizer compounds PF-07304814 (the parent compound PF-00835231), 10 11a, GC-376, 11b the deuterated GC-376 (D2-GC-376), 5, 6j, 11a MI-09, MI-30, 3 and MPI8 (Figure 1). Although the high reactivity of these reactive warheads, especially the aldehyde, confers potent activities in the enzymatic assay and antiviral assay, it inevitably leads to off-target side effects through reaction with some host proteins. 16-20 For example, we and others have shown that GC-376 is a potent inhibitor of cathepsin L (Table 1). 17,20 A recent study revealed that MPI8, an analogue of GC-376 with an aldehyde warhead, inhibits cathepsins B, L, and K with IC_{50} values of 1.2, 230, and 180 nM, respectively. 15 The off-target effect is also a potential concern for some of the most advanced Mpro inhibitors including the clinical candidate PF-07304814, 21 compounds 6j and 6e which showed in vitro antiviral efficacy against MERS-CoV-2 infection in mice, and compound 11a with potent in vitro antiviral activity (Table 1). 22 All of these compounds are potent inhibitors of cathepsin L. The high reactivity of the aldehyde warhead might confer the lack of target specificity, and the design of covalent inhibitors with a high target specificity remains a daunting task.

We report herein the rational design of covalent Mpro inhibitors with novel cysteine reactive warheads and high target specificity. Specifically, guided by the X-ray crystal structure of SARS-CoV-2 Mpro with 23R (Jun8-76-3A) (PDB code 7KXS), which was one of the most potent noncovalent Mpro inhibitors developed from our earlier study, 23 we systematically explored a number of novel electrophiles in the replacement of the P1′ furyl substitution in 23R. The aim is to identify C145 reactive electrophiles with both potent Mpro inhibition and high target selectivity. This effort led to the discovery of several novel cysteine reactive warheads including dichloroacetamide, dibromoacetamide, tribromoacetamide, 2-bromo-2,2-dichloroacetamide, and 2-chloro-2,2-dibromoacetamide. One of the most potent lead compounds Jun9-62-2R (dichloroacetamide) inhibited SARS-CoV-2 Mpro with an IC_{50} of 0.43 μM and viral replication with an EC_{50} of 2.05 μM in Caco2-hACE2 cells. Significantly, unlike GC-376, Jun9-62-2R (dichloroacetamide) and Jun9-88-6R (tribromoacetamide) are highly selective toward Mpro and do not inhibit the host calpain I, cathepsins B, K, L, caspase-3, and trypsin. X-ray crystal structure of SARS-CoV-2 Mpro with Jun9-62-2R (dichloroacetamide) and Jun9-88-6R (tribromoacetamide) revealed that the C145 forms a covalent adduct with the reactive warheads. Overall, the discovery of these di- and trihaloacetamides as novel cysteine reactive warheads sheds light on the feasibility of developing SARS-CoV-2 Mpro inhibitors with high target specificity over tested calpain and cathepsins and a high cellular selectivity index. These novel compounds represent

![Figure 1. AdvancedSARS-CoV-2 Mpro inhibitors with translational potential.](https://doi.org/10.1021/jacs.1c08060)

**Table 1. Target Specificity of SARS-CoV-2 Mpro Inhibitors**

| compd | SARS-CoV-2 Mpro, IC_{50} (nM) | cathepsin L, IC_{50} (nM) | additional off targets | ref |
|-------|-----------------------------|--------------------------|------------------------|-----|
| GC-376 | 33 | 0.99 | calpain I (IC_{50} = 74 nM) | 8, 17, 18, 20, 23 |
| MPI8 | 105 | 1.2 | cathepsin K (IC_{50} = 0.56 nM) | 15, 16 |
| PF-00835231 | 5 | 146 | cathepsin B (IC_{50} = 230 nM) | 19, 21 |
| 6e | 10 | <0.5 | | 19 |
| 6j | 7 | <0.5 | | 19 |
| 11a | 8 | 0.21 | cathepsin B (IC_{50} = 1.3 μM) | 19, 22 |
valuable chemical probes for target validation and drug candidates for further development as SARS-CoV-2 antivirals.

**RESULTS AND DISCUSSION**

**Synthesis of Covalent M<sup>pro</sup> Inhibitors.** The covalent M<sup>pro</sup> inhibitors were synthesized by the one-pot Ugi four-component reaction (Ugi-4CR) as shown for Jun9-62-2 (Figure 2) with yields from 33% to 88%. For compounds with potent enzymatic inhibition, the diastereomers were subsequently separated by chiral HPLC. The absolute stereochemistry of Jun9-57-3R and Jun9-62-2R was determined by X-ray crystallography, and the stereochemistry for the diastereomers of Jun9-90-4, Jun9-89-2, Jun9-89-4, and Jun9-88-6 was tentatively assigned based on their relevant retention times in chiral HPLC.

**Rational Design of Covalent M<sup>pro</sup> Inhibitors.** 23R was designed based on the superimposed X-ray crystal structure of GC-376 with ML188 and UAWJ254.23,24 The X-ray crystal structure showed that the furyl substitution at the P1′ position of 23R is in close proximity with the catalytic cysteine 145 (3.4 Å between C145 sulfur and the C-2 carbon of furyl, PDB code 7KXS) (Figure 3A), suggesting that replacement of furyl with a reactive warhead might lead to covalent inhibitors (Figure 3B). 23R is an ideal lead candidate for the design of covalent M<sup>pro</sup> inhibitors for several reasons: (1) the P1, P2, and P3 substitutions have already been optimized; (2) the designed compounds can be expeditiously synthesized by the one-pot Ugi-4CR; (3) a diversity of cysteine reactive warheads are commercially available and can be promptly introduced at the P1′ position to react with the C145.

Although a number of thiol-reactive warheads have been developed in the field of covalent protease and kinase inhibitors,25–27 we decided to focus on pharmacologically compliant reactive warheads from the FDA-approved drugs. The majority of FDA-approved thiol-reactive drugs are kinase inhibitors including ibrutinib, osimertinib, zanubrutinib, acalabrutinib, dacomitinib, neratinib, and afatinib (Figure 3C).25 As such, acrylamide and 2-butynamide were chosen as reactive warheads in our initial design of covalent SARS-CoV-2 M<sup>pro</sup> inhibitors (Figure 3B). Chloroacetamide was also chosen as it was previously explored by Pfizer for the development of SARS-CoV and SARS-CoV-2 M<sup>pro</sup> inhibitors (Pfizer compound 12) (Figure 3C).21 Chloroacetamide is frequently used as a reactive warhead for designing chemical probes for target pull down.28 Finally, we included azido-methylene as it was previously shown to be a relatively unreactive cysteine warhead.29,30 The fluoroacetamide was included as a control.

The designed covalent SARS-CoV-2 M<sup>pro</sup> inhibitors were shown in Figure 3D. All compounds were first tested in the FRET-based M<sup>pro</sup> enzymatic assay. Active hits were further tested for cellular cytotoxicity to select candidates for the following antiviral assay against SARS-CoV-2. It was found that the azidoacetamide Jun9-61-1 and the fluoracetamide Jun9-61-4 were not active (IC<sub>50</sub> > 20 µM). Surprisingly, the acrylamides Jun10-15-2 and Jun9-51-3 were also not active (IC<sub>50</sub> > 20 µM), suggesting the acrylamide might not be positioned at the right geometry for reacting with the C145. Gratifyingly, Jun9-62-1 with the 2-butynamide warhead showed potent inhibition with an IC<sub>50</sub> of 1.15 µM. However, Jun9-62-1 also had moderate cytotoxicity in both Vero E6 (CC<sub>50</sub> = 17.99 µM) and Calu-3 (CC<sub>50</sub> = 47.77 µM) cells. Similarly, covalent inhibitors with the chloroacetamide reactive warhead had potent inhibition against SARS-CoV-2 M<sup>pro</sup>. The most potent compound Jun9-57-3R inhibited SARS-CoV-2 M<sup>pro</sup> with an IC<sub>50</sub> of 0.05 µM, comparable to the potency of GC-376 (IC<sub>50</sub> = 0.03 µM). Interestingly, the diastereomer Jun9-57-3S was also a potent M<sup>pro</sup> inhibitor with an IC<sub>50</sub> of 1.13 µM. However, covalent inhibitors with the chloroacetamide warhead Jun9-54-1, Jun9-59-1, Jun9-55-2, Jun9-57-3R, Jun9-57-3S, Jun9-57-2, and Jun9-55-1 were highly cytotoxic in Vero E6 (CC<sub>50</sub> < 11 µM) and Calu-3 (CC<sub>50</sub> < 2 µM) cells, possibly due to their off-target effects on host proteins/DNAs. The low cellular selectivity index precludes further development of these covalent M<sup>pro</sup> inhibitors as SARS-CoV-2 antiviral drugs.

**Exploring Acrylamides and Haloacetamides as Novel Warheads for SARS-CoV-2 M<sup>pro</sup> C145.** For the acrylamide series of compounds, Jun9-72-3 and Jun10-31-4, both containing a 2-substituted acrylamide warhead, were not active against M<sup>pro</sup> (IC<sub>50</sub> > 20 µM) (Figure 4). However, compound Jun10-38-2 with the 2-chloroacrylamide had potent inhibition with an IC<sub>50</sub> of 4.22 µM. For the haloacetamide series of compounds, the reference compound Jun9-54-1 with the classical chloroacetamide reactive warhead had potent inhibition against SARS-CoV-2 M<sup>pro</sup> with an IC<sub>50</sub> of 0.17 µM. However, it was cytotoxic in both Vero E6 cells and Calu-3 cells with CC<sub>50</sub> values less than 3.5 µM. To increase the cellular selectivity index, we reasoned that substituted chloroacetamides or haloacetamides might have reduced cellular cytotoxicity while maintaining potent M<sup>pro</sup> inhibition. It was found that Jun9-77-1 with the 2-chloropropanamide warhead was not active (IC<sub>50</sub> > 20 µM). Encouragingly, compound Jun9-62-2R with the dichloroacetamide warhead had potent inhibition against M<sup>pro</sup> with an IC<sub>50</sub> of 0.43 µM while being noncytotoxic to Vero E6 cells (CC<sub>50</sub> > 100 µM). In comparison, the corresponding diastereomer Jun9-62-2S was not active (IC<sub>50</sub> > 20 µM), which is consistent with the predicted binding mode (Figure 3A). Given these promising results, we further designed two...
additional dichloroacetamide compounds \textit{Jun9-90-3} and \textit{Jun9-90-4} with variations at the P3/P4 substitutions. Similar to \textit{Jun9-62-2R}, both \textit{Jun9-90-3R} and \textit{Jun9-90-4R} were potent inhibitors with IC\textsubscript{50} values of 0.30 and 0.46 \(\mu\text{M}\), respectively. Both compounds were also noncytotoxic to Vero E6 cells (CC\textsubscript{50} > 100 \(\mu\text{M}\)). In contrast, the corresponding diastereomers \textit{Jun9-90-3S} and \textit{Jun9-90-4S} were not active (IC\textsubscript{50} > 20 \(\mu\text{M}\)).
We further explored di- and trisubstituted haloacetamides as M\textsuperscript{pro} C\textsubscript{145} reactive warheads (Figure 4). Jun\textsubscript{9}-89-2R with the dibromoacetamide warhead is highly active with an IC\textsubscript{50} of 0.08 μM; however, the cell cytotoxicity also increased (CC\textsubscript{50} = 8.94 μM). The diastereomer Jun\textsubscript{9}-89-2S also had potent inhibition against M\textsubscript{pro} with an IC\textsubscript{50} of 2.44 μM and comparable cytotoxicity (CC\textsubscript{50} = 4.57 μM). Jun\textsubscript{9}-76-4 with the 2,2-dichloropropanamide warhead, Jun\textsubscript{9}-72-4 with the trichloroacetamide, and Jun\textsubscript{9}-77-2 with the 2-chloro-2,2-difluoroacetamide were all inactive against M\textsubscript{pro} (IC\textsubscript{50} > 20 μM). Jun\textsubscript{9}-89-3 with the 2-bromo-2,2-dichloroacetamide showed potent inhibition with an IC\textsubscript{50} of 1.20 μM. The cytotoxicity of Jun\textsubscript{9}-89-3 also improved (CC\textsubscript{50} = 32.43 μM). Jun\textsubscript{9}-72-4 with the 2-chloro-2,2-dibromoacetamide warhead is highly potent with an IC\textsubscript{50} of 0.05 μM, but it was cytotoxic in Vero E6 cells (CC\textsubscript{50} = 8.41 μM). The diastereomer Jun\textsubscript{9}-89-4S was less active (IC\textsubscript{50} = 0.04 μM). Jun\textsubscript{9}-88-6R with the tribromoacetamide warhead had high potency against M\textsubscript{pro} with an IC\textsubscript{50} of 0.08 μM, while the diastereomer Jun\textsubscript{9}-88-6S was less active (IC\textsubscript{50} = 0.76 μM). Both Jun\textsubscript{9}-88-6R and Jun\textsubscript{9}-88-6S had comparable cytotoxicity as Jun\textsubscript{9}-54-1 with CC\textsubscript{50} values of 5.48 and 5.99 μM, respectively.

**Pharmacological Characterization of SARS-CoV-2 M\textsubscript{pro} Inhibitors with Novel Reactive Warheads.** On the basis of the M\textsubscript{pro} inhibition and cell cytotoxicity, four compounds Jun\textsubscript{9}-62-2R, Jun\textsubscript{9}-90-3R, Jun\textsubscript{9}-90-4R, and Jun\textsubscript{9}-88-6R were selected for mechanistic studies (Figure 5). Enzymatic kinetic studies suggested that these four compounds bind to M\textsubscript{pro} in a two-step process: the first step is reversible binding (K\textsubscript{I}), and the second step is irreversible binding (k\textsubscript{inact}). The calculated k\textsubscript{inact}/K\textsubscript{I} values for Jun\textsubscript{9}-62-2R, Jun\textsubscript{9}-90-3R, Jun\textsubscript{9}-90-4R, and Jun\textsubscript{9}-88-6R were 189.7, 1543.6, 867.4, and 7074.3 M\textsuperscript{-1} s\textsuperscript{-1}, respectively (Figure 5A). These results were in agreement with the expected mechanism of
Figure 5. Pharmacological characterization of the SARS-CoV-2 M\textsuperscript{pro} inhibitors. (A) Curve fittings of the enzymatic kinetic studies of four compounds Jun9-62-2R, Jun9-90-3R, Jun9-90-4R, and Jun9-88-6R against SARS-CoV-2 M\textsuperscript{pro}. (B) Binding of four compounds Jun9-62-2R, Jun9-90-3R, Jun9-90-4R, and Jun9-88-6R to SARS-CoV-2 M\textsuperscript{pro} in the thermal shift assay. (C) Fast dilution experiment. 10 μM M\textsuperscript{pro} was preincubated with 10 μM testing compounds for 2 h at 30 °C; the preformed compound–enzyme complex was diluted 100-fold into reaction buffer before initiation of the enzymatic reaction. The recovered enzymatic activity was compared with DMSO control. 23R is a noncovalent M\textsuperscript{pro} inhibitor, and it was included as a control. (D) Time dependent inhibition of M\textsuperscript{pro} by Jun9-62-2R. 100 nM SARS CoV-2 M\textsuperscript{pro} was preincubated with Jun9-62-2R for various periods of time (0 min to 2 h) before the addition of 10 μM FRET substrate to initiate the enzymatic reaction. 23R was included as a control. (E–H) Native mass spectrometry assay of SARS-CoV-2 M\textsuperscript{pro} reveals binding of Jun9-62-2R with mass modifications of...
action in which all four compounds form a covalent bond with the catalytic C145. In the thermal shift-binding assay, all four compounds stabilized the SARS-CoV-2 MPro upon binding as reflected by the $T_m$ shift to higher temperatures (Figure 5B).
As the tribromoacetamide is sterically hindered, the mechanism of action of Jun9-88-6R might involve the nucleophilic attack of the carbonyl by the C14S thiol to give a thiohemiketal intermediate, followed by a 1,2-shift of the sulfur to displace one bromide (Figure S2).

To provide additional lines of evidence to support the proposed mode of action of covalent binding, we performed three additional experiments. First, to demonstrate the reversibility of the binding of Jun9-62-2R to Mpro, we incubated 10 μM SARS-CoV-2 Mpro with 10 μM Jun9-62-2R for 2 h and monitored the enzymatic activity of Mpro following 100-fold dilution of the mixture. It was found that no enzymatic activity was recovered (Figure 5C). In contrast, the mixture with our previously developed noncovalent inhibitor 23R showed nearly complete recovery of enzymatic activity after dilution (Figure 5C). These results suggest that the binding of Jun9-62-2R is irreversible while the binding of 23R is reversible. Second, we repeated the FRET assay of Jun9-62-2R with different preincubation times and found that longer preincubation time gave lower IC50 values (Figure 5D). These data are consistent with the mode of action of covalent inhibitors. In contrast, preincubation of Mpro with the noncovalent inhibitor 23R did not lead to significant changes of the IC50 value (Figure 5D). Third, we used native mass spectrometry to detect the covalent adducts of Mpro with Jun9-62-2R, Jun9-89-2R, Jun9-88-6R, and Jun9-89-4R. The expected mass shifts of 482 Da and 526 Da were observed for Jun9-62-2R and Jun9-89-2R, respectively (Figure 5E,F). Interestingly, the expected dibromoacetamide conjugate was not observed for Jun9-88-6R, suggesting this conjugate might not be stable. Instead, the mass shift corresponding to the monobromo thiol adduct was observed (Figure 5G). For Jun9-89-4R, the mass shifts for both the chlorobromo and chloro thiol adducts were observed (Figure 5H).

To further profile the cellular Mpro inhibition, we tested these four compounds in our recently developed FlipGFP assay. Briefly, the GFP is split into two parts, the β1−9 template and the β10−11 strands. The β10 and β11 strands were engineered with KS-E5 linker such that they are restrained in the parallel form. When the linker is cleaved by Mpro, β10 and β11 adopt antiparallel conformation, which allows association with the β1−9 template, leading to the recovery of the GFP signal. In the FlipGFP assay, the GFP signal is proportional to the Mpro enzymatic activity. It was found that all four compounds led to dose-dependent inhibition of the GFP signal with EC50 values of 0.96 μM (Jun9-62-2R), 0.91 μM (Jun9-90-3R), 1.57 μM (Jun9-90-4R), and 0.92 μM (Jun9-88-6R) (Figure 5I,J). The EC50 value for the positive control GC-376 was 1.80 μM. This result suggests that these four compounds can potently inhibit the Mpro in the cellular content.

**Antiviral Activity of SARS-CoV-2 Mpro Inhibitors with Novel Reactive Warheads.** The antiviral activity of the four lead compounds was evaluated in both Vero E6 cells and Caco2-hACE2 cells to exclude cell type dependent effect. Caco2-hACE2 with endogenous TMPRSS2 expression is a validated cell line for SARS-CoV-2 antiviral assay. Jun9-62-2R, Jun9-90-3R, Jun9-90-4R, and Jun9-88-6R inhibited SARS-CoV-2 replication in Vero E6 cells with EC50 values of 0.90, 2.07, 1.10, and 0.58 μM, respectively (Figure 6A). All four compounds showed comparable antiviral activity in Caco2-hACE2 cells with EC50 values of 2.05, 3.24, 1.43, and 2.15 μM, respectively (Figure 6B). In comparison, GC-376 inhibited SARS-CoV-2 replication in Vero E6 and Caco2-hACE2 cells with EC50 values of 1.51 and 2.90 μM. When tested in Calu-3 cells, Jun9-90-3R showed comparable antiviral activity with an EC50 value of 2.00 μM (Figure 6C).

**Profiling the Target Selectivity against Host Proteases.** Lack of target specificity is one of the major reasons that many cysteine protease inhibitors failed in the clinical trials. To profile the target specificity of these SARS-CoV-2 Mpro inhibitors with a novel reactive warhead, we selected Jun9-62-2R and Jun9-88-6R as representative examples and included the canonical GC-376 with an aldehyde reactive warhead for comparison. The results showed that GC-376 had potent inhibition of the host proteases including calpain I, calpain II, cathepsin B, cathepsin K, cathepsin L, caspase-3, and trypsin.
cathepsin B, cathepsin K, and cathepsin L with IC$_{50}$ values in the submicromolar and nanomolar range. GC-376 did not inhibit caspase-3 and trypsin (IC$_{50} > 20$ μM) (Figure 7). In comparison, both Jun9-62-2R and Jun9-88-6R had a significantly improved target selectivity and did not show potent inhibition against the host calpain 1, cathepsin B, cathepsin K, cathepsin L, caspase-3, and trypsin. Jun9-88-6R had weak inhibition against cathepsin L with an IC$_{50}$ of 7.37 μM, conferring a 94-fold higher selectivity for inhibiting the SARS-CoV-2 Mpro. Collectively, the covalent SARS-CoV-2 Mpro inhibitors Jun9-62-2R with the dichloroacetamide warhead and Jun9-88-6R with the tribromoacetamide warhead have high target selectivity against Mpro over host proteases.

Figure 8. X-ray crystal structures of SARS-CoV-2 Mpro in complex with Jun9-62-2R (A) and Jun9-57-3R (B). 2Fo – Fc electron density map, shown in gray, is contoured at 1σ. Structural superpositions of the noncovalent analogues Jun8-76-3A (white, PDB code 7KX5) and ML188 (yellow, PDB code 7L0D) with Jun9-62-2R (C) and Jun9-57-3R (D) reveal a different mode of interaction with the catalytic core.

cathpsin B, cathpsin K, and cathpsin L with IC$_{50}$ values in the submicromolar and nanomolar range. GC-376 did not inhibit caspase-3 and trypsin (IC$_{50} > 20$ μM) (Figure 7). In comparison, both Jun9-62-2R and Jun9-88-6R had a significantly improved target selectivity and did not show potent inhibition against the host calpain 1, cathepsin B, cathepsin K, cathepsin L, caspase-3, and trypsin. Jun9-88-6R had weak inhibition against cathepsin L with an IC$_{50}$ of 7.37 μM, conferring a 94-fold higher selectivity for inhibiting the SARS-CoV-2 Mpro. Collectively, the covalent SARS-CoV-2 Mpro inhibitors Jun9-62-2R with the dichloroacetamide warhead and Jun9-88-6R with the tribromoacetamide warhead have high target selectivity against Mpro over host proteases.

X-ray Crystal Structures of SARS-CoV-2 Mpro in Complex with Jun9-62-2R and Jun9-57-3R. Using X-ray crystallography, we solved the complex structures of SARS-CoV-2 Mpro with Jun9-57-3R (2.25 Å, PDB code 7RN0) and Jun9-62-2R (2.30 Å, PDB code 7RN1) (Figure 8, Table S1). Jun9-57-3R and Jun9-62-2R have nearly identical chemical features to their noncovalent progenitor 23R (Jun8-76-3A) (PDB code 7KX5). As such, the binding poses are very similar. The pyridyl ring binds to the S1 pocket of Mpro, where it forms a hydrogen bond with His163. This hydrogen bond is critical for coordinating the Gln side chain of its substrate, a residue it is uniquely selective for. Consequently, a hydrogen bond acceptor at this position confers tremendous potency to Mpro inhibitors. The phenylpyrrole (Jun9-57-3R) or biphenyl (Jun9-62-2R) moieties insert into the hydrophobic S2 pocket where they form nonpolar contacts and stack with the catalytic base, His41. An amide group linking the pyridyl ring to an α-methylbenzene group accepts a hydrogen bond from the main chain of Glu166. This α-methylbenzene group flips down toward the core of the substrate channel, where it forms additional π-stacking interactions with the biphenyl or phenylpyrrole moieties. The key distinction between Jun9-62-2R, Jun9-57-3R, and analogues Jun8-76-3A and ML188 is the presence of an electrophilic chloroacetamide warhead, which forms a covalent adduct with the catalytic cysteine Cys145 (Figure 8C,D). The short distance of this covalent bond (1.8 Å) allows the inhibitor to press further into the
oxyanion hole, causing the P2 benzene to rotate inward by ~40°. Likewise, the chloracetamide warhead is forced toward the catalytic core, causing the P1’ chloride of Jun9-57-3R to lie closer to Cys145 (2.8 Å) than the corresponding furyl oxygen of Jun8-76-3A (3.2 Å).

## CONCLUSION

The majority of the reported Mpro inhibitors contain the aldehyde reactive warhead, which is known to have nonspecific reactivity toward host proteins.16-19 It should be noted that both of the Pfizer Mpro inhibitors that are currently in clinical trials do not contain the aldehyde warhead.9,10 As such, we are interested in developing SARS-CoV-2 Mpro inhibitors with high target specificity. A highly specific Mpro inhibitor is also needed for target validation as it separates the effect of Mpro inhibition from host protease inhibition such as cathepsin L. It is known that host cathepsin L is important in SARS-CoV-2 replication in Vero E6 cells, which are TMPRSS2-negative, but not in Calu-3 cells, which are TMPRSS2-positive.16 In this study, we report the discovery of dichloroacetamide, di- and trihaloacetamide-containing Mpro inhibitors as novel cysteine reactive warheads. To the best of our knowledge, these warheads have not been explored in cysteine protease inhibitors. The most promising lead compounds Jun9-62-2R with the dichloroacetamide warhead and Jun9-88-6R with the tribromoacetamide inhibited SARS-CoV-2 Mpro with IC50 values of 0.43 μM and 0.08 μM, respectively. These two compounds also showed potent inhibition against SARS-CoV-2 in both Vero E6 and Caco2-hACE2 cells with EC50 values in the single-digit micromolar to submicromolar range. Significantly, both Jun9-62-2R and Jun9-88-6R had high target specificity toward Mpro and did not inhibit the host proteases including calpain I, cathepsin B, cathepsin K, cathepsin L, caspase-3, and trypsin. In comparison, GC-376 was not selective and inhibited calpain I, cathepsin B, cathepsin K, and cathepsin L with comparable potency as Mpro. Regarding the translational potential of the di- and trihaloacetamide-containing Mpro inhibitors, the widely used antibiotic chloramphenicol contains the dichloroacetamide, suggesting Jun9-62-2R might be tolerated in vivo. Follow-up studies will optimize the in vitro and in vivo pharmacokinetic properties and in vivo antiviral efficacy of these novel compounds in SARS-CoV-2 infection animal models. Other potential strategies include developing selective Mpro inhibitors including allostERIC inhibitors37,38 or targeting the more reactive Cys44 at the S2 binding pocket.39,40 Overall, these novel compounds represent valuable chemical probes for target validation and promising drug candidates for translational development as SARS-CoV-2 antivirals.

## ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c08060. Additional figures and tables describing the experimental materials, methods, X-ray data set, synthesis, and characterization of the Mpro inhibitors (PDF)

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Notes
The authors declare the following competing financial interest(s): Jun Wang is an inventor of a filed patent claiming the use of \textit{Jun9-62-2R} and related compounds as potential COVID-19 antiviral drugs.

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\section*{References}
(1) Harvey, W. T.; Carabelli, A. M.; Jackson, B.; Gupta, R. K.; Thomson, E. C.; Harrison, E. M.; Ludden, C.; Reeve, R.; Rambaut, A.; Peacock, S. J.; Robertson, D. L.; COVID-19 Genomics UK (COG-UK) Consortium.. SARS-CoV-2 variants, spike mutations and immune escape. \textit{Nat. Rev. Microbiol.} \textbf{2021}, \textit{19} (7), 409–424.

(2) Cox, R. M.; Wolf, J. D.; Plumper, R. K. Therapeutically administered ribonucleoside analogue MK-4482/EIDD-2801 blocks SARS-CoV-2 transmission in ferrets. \textit{Nat. Microbiol.} \textbf{2021}, \textit{6} (1), 11–18.

(3) Morose, 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. \textit{ChemBioChem} \textbf{2020}, \textit{21} (5), 730–738.

(4) 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, Y.; 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, W. G.; Li, W. M.; Wei, Y. Q.; Zheng, Y. T.; Lei, J.; Yang. SARS-CoV-2 M(pro) inhibitors with antiviral activity in a transgenic mouse model. \textit{Science} \textbf{2021}, \textit{371} (6536), 1374–1378.

(5) Dampalla, C. S.; Zheng, J.; Perera, K. D.; Wong, L. R.; Meyerholz, D. K.; Nguyen, H. N.; Kashipathy, M. M.; Battaile, K. P.; Lovell, S.; Kim, Y.; Perlman, S.; Groutas, W. C.; Chang, K. O. Postinfection treatment with a protease inhibitor increases survival of mice with a fatal SARS-CoV-2 infection. \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{2021}, \textit{118} (29), No. e2101555118.

(6) Caceres, C. J.; Cardenas-Garcia, S.; Carnaccini, S.; Seibert, B.; Rajao, D. S.; Wang, J.; Perez, D. R. Efficacy of GC-376 against SARS-CoV-2 virus infection in the K18 HACE2 transgenic mouse model. \textit{Sci. Rep.} \textbf{2021}, \textit{11} (1), 9609.

(7) Ma, C.; Sacco, M. D.; Huest, 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 XII inhibit SARS-CoV-2 viral replication by targeting the viral main protease. \textit{Cell Res.} \textbf{2020}, \textit{30} (8), 678–679.
advantages of describing covalent inhibitor in vitro potencies by IC50 at a fixed time point. IC50 determination of covalent inhibitors provides meaningful data to medicinal chemistry for SAR optimization. Bioorg. Med. Chem. 2021, 29, 118565.

(32) Drayman, N.; DeMarco, J. K.; Jones, K. A.; Azizi, S.-A.; Froggatt, H. M.; Tan, K.; Maltesa, N. I.; Chen, S.; Nicolaeescu, V.; Dvorkin, S.; Furlong, K.; Kathayat, R. S.; Firpo, M. R.; Mastrodomenico, V.; Bruce, E. A.; Schmidt, M. M.; Jedrzejczak, R.; Munoz-Alia, M. A.; Schuster, B.; Nair, V.; Han, Y.-Y.; O’Brien, A.; Tomatisidou, A.; Meyer, B.; Vignuzzi, M.; Missiakas, D.; Botton, J. W.; Brooke, C. B.; Lee, H.; Baker, S. C.; Mounce, B. C.; Heaton, N. S.; Severson, W. E.; Palmer, K. E.; Dickinson, B. C.; Joachimaki, A.; Randall, G.; Tay, S. Masitinib is a broad coronavirus 3CL inhibitor that blocks replication of SARS-CoV-2. Science 2021, 373 (6557), 931−936.

(33) Hoffmann, M.; Kleine-Weber, H.; Schroeder, S.; Kruger, N.; Herrler, T.; Eriuchen, S.; Schiergens, T. S.; Herrler, G.; Wu, N. H.; Nitsche, A.; Muller, M. A.; Drosten, C.; Pohmann, 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.

(34) Bertram, S.; Glowacka, I.; Blazewijska, P.; Soilleux, E.; Allen, P.; Choch, S.; Steffen, M.; Y. S. Y.; Park, Y.; Schneider, H.; Schughart, K.; Pohmann, S. TMPRSS2 and TMRPSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. J. Virol. 2010, 84 (19), 10016−25.

(35) Stanifer, M. L.; Kee, C.; Cortese, M.; Zumaran, C. M.; Triana, S.; Mukenhirn, M.; Kraeuessich, H. G.; Alexandrov, T.; Bartenschlager, R.; Boulant, S. Critical Role of Type III Interferon in Controlling SARS-CoV-2 Infection in Human Intestinal Epithelial Cells. Cell Rep. 2020, 32 (1), 107863.

(36) Shang, J.; Wan, Y.; Luo, C.; Ye, G.; Geng, Q.; Auerbach, A.; Li, F. Cell entry mechanisms of SARS-CoV-2. Proc. Natl. Acad. Sci. U. S. A. 2020, 117 (21), 11277−11278.

(37) Gunther, S.; Reine, P. Y. A.; Fernandez-Garcia, Y.; Liesz, J.; Lane, T. J.; Ginn, H. M.; Koua, F. H. M.; Ehrt, C.; Ewert, W.; Oberthuer, D.; Yefanov, O.; Meier, S.; Lorenzen, K.; Krichel, B.; Kopicki, J. D.; Gelsio, L.; Brehm, W.; Dunkel, I.; Seychell, B.; Gribbon, P.; Welder, J.; Serebryan, V.; Stoycheva, A.; Chanda, S.; Beigelman, L.; Blatt, L. M.; Boland, S.; Vangeel, L.; Dejonghe, S.; Chaltin, P.; Marchand, A.; Serebryan, V.; Stoycheva, A.; Chanda, S.; Symons, J. A.; Raboisson, P.; Nitsche, A.; Muller, M. A.; Drosten, C.; Pohmann, S. SARS-CoV-2 Main Protease Inhibitors Guided by the Superimposed SARS-CoV-2 Main Protease. J. Med. Chem. 2020, 63 (21), 12725−12747.

(38) Jacob, T.; Grum-Tokares, V.; Zhou, Y.; Turlington, M.; Saldanha, S. A.; Chase, P.; Eggerl, A.; Dawson, E. S.; Baez-Santos, M. M.; Tomar, S.; Mielech, A. M.; Baker, S. C.; Lindsay, C. W.; Hepler, P.; Meseac, A.; Stauffer, S. D.; Discovery, synthesis, and structure-based optimization of a series of N-(tert-butyl)-2-(pyrindin-3-yl) acetonitriles (ML188) as potent non-covalent small molecule inhibitors of the severe acute respiratory syndrome coronavirus (SARS-CoV) 3CL protease. J. Med. Chem. 2013, 56 (2), 534−546.

(39) Abdeldaim, A.; Raouf, Y. S.; Constantinescu, S. N.; Moriggl, R.; Gunning, P. T. Advances in covalent kinase inhibitors. Chem. Soc. Rev. 2020, 49 (9), 2617−2687.

(40) Siklos, M.; BenAssia, M.; Thatcher, G. R. Cysteine proteases as therapeutic targets: do selectivity matter? A systematic review of capathine and capathisin inhibitors. Acta Pharm. Sin. B 2015, 5 (6), 506−519.

(41) Ciani, L.; Feldmann, C. W.; Gilberg, E.; Gutschow, M.; Juliano, L.; Leitao, A.; Bajorath, J.; Montanari, C. A. Can Cysteine Protease Cross-Class Inhibitors Achieve Selectivity? J. Med. Chem. 2019, 62 (23), 10497−10525.

(42) Hoch, D. G.; Abegg, D.; Adibekian, A. Cysteine-reactive probes and their use in chemical proteomics. Chem. Commun. 2018, 54 (36), 4501−4512.

(43) Le, G. T.; Abbenante, G.; Madala, P. K.; Hoang, H. N.; Fairlie, D. P. Organic Azide Inhibitors of Cysteine Proteases. J. Am. Chem. Soc. 2006, 128 (38), 12386−12397.

(44) Yang, P.-Y.; Wu, H.; Lee, M. Y.; Xu, A.; Srinivasan, R.; Yao, S. Q. Solid-Phase Synthesis of Azidomethylene Inhibitors Targeting Cysteine Proteases. Org. Lett. 2008, 10 (10), 1881−1884.

(45) Thorarensen, A.; Balbo, P.; Banker, M. E.; Czerwinski, R. M.; Kuhn, M.; Maurer, T. S.; Telliez, J.-B.; Vincent, F.; Wittwer, A. J. The
Verma, N.; Henderson, J. A.; Shen, J. Proton-Coupled Conformational Activation of SARS Coronavirus Main Proteases and Opportunity for Designing Small-Molecule Broad-Spectrum Targeted Covalent Inhibitors. *J. Am. Chem. Soc.* **2020**, *142* (52), 21883−21890.