Coronaviruses (CoVs) are dangerous and widespread pathogens that cause a plethora of diseases in many species of animals and humans. The betacoronavirus includes severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2, which causes the COVID-19 disease. Over the past two decades, coronaviruses have been responsible for three major pandemic outbreaks by crossing the species barrier and causing serious respiratory infection in humans: the 2003 SARS-CoV, 2012 Middle East respiratory syndrome coronavirus (MERS-CoV), and 2019 COVID-19. Because of a rapid increase in cases worldwide, the World Health Organization (WHO) declared COVID-19 a pandemic in March 2020. Two years later, the disease has infected over 456 million people and caused over 6 million deaths worldwide. Currently, its management includes social distancing, face-covering, sanitation, isolation, supportive care, treatment of symptoms, immunizations, and antiviral treatments.

The coronavirus encodes for two cysteine proteases, the papain-like cysteine protease (PLpro) and the chymotrypsin-like cysteine or main protease (Mpro). These enzymes catalyze the proteolysis of two large overlapping viral polyproteins (pp1a and pp1ab) that are involved in the production of the replicase complex mediating viral replication and transcription. Mpro is a ~33 kDa cysteine protease that catalyzes the replicase polyprotein at 11 conserved sites by hydrolyzing the Leu-Gln bond (Ser, Ala, and Gly) recognition sequence. Furthermore, it was one of the first SARS-CoV-2 proteins to have its 3-D structure solved by X-ray crystallography. Mpro is a homodimer with two subunits organized perpendicular to each other. The two protomers are connected by Ser1 of one protomer to the Phe140 and Glu166 of the other protomer. The dimerization of the protease structure, while Domain III (Thr201-Glu507) comprises five α-helices and helps in the enzyme dimerization. Domains II and III are connected by a flexible loop region (Phe185-Ile200). The folding scaffolds of Domains I and II are similar to other viral chymotrypsin-like proteases. Each protomer contains an active site composed of a His41−Cys145 catalytic dyad at the juncture between Domain I and II. Interestingly, Mpro contains Cys catalytic residues, unlike other chymotrypsin-like enzymes. This active site has canonical binding pockets named S1, S1′, S2, and S3 for accommodating the P1, P1′, P2, and P3 residues of the endogenous substrate peptide. A proposed catalytic mechanism supported by mixed quantum mechanical and molecular mechanical (QM/MM) simulations postulated that the cleavage of the peptide bond takes place in a stepwise manner. Initially, the protein substrate attaches to the binding pocket and creates an enzyme−substrate complex.

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Simultaneously, the negative sulfur atom of Cys145 makes a nucleophilic attack on the carbonyl carbon atom of the peptide bond and causes the formation of a stable thiohemiketal intermediate. In the second step, the positively charged His41 transfers a proton to the amide nitrogen atom, resulting in the cleavage of the scissile peptide bond and release of the C terminal cleavage product. At the last step, a water molecule hydrolyzes the thioester between the cysteine and part of the substrate, forming the other cleavage product formation of the product.30

Currently, out of ~350 compounds undergoing clinical trials, remdesivir (RdRp inhibitor), molnupiravir (RdRp inhibitor),31 and nirmatrelvir (Mpro inhibitor)32 are the only U.S. Food and Drug Administration (FDA)-authorized drugs available for the treatment of COVID-19.32,33 The current pharmacotherapy utilized by the FDA is allocated into three main types: (1) antiviral therapy, (2) immune-based therapy, and (3) immunomodulators. For example, remdesivir comes under antiviral therapy, while human blood-derived products and plasma are used for immune-based therapy, and corticosteroids like dexamethasone are used as an immunomodulatory agent.33−38 Among these strategies, antiviral therapy is most promising when used during the earliest stages of the disease, whereas immune-based and immunomodulating therapies are more effective in the later phase of the ailment.39−41 Nevertheless, new antiviral therapies that inhibit the viral replication by either inhibiting entry or by acting on the 3CLpro and PLpro enzymes are urgently needed to combat this devastating disease and reduce the potential for hospitalization.

As a consequence of the previous MERS and SARS outbreaks, various Mpro inhibitors have been proposed experimentally and computationally. For instance, in 2008, Nukoolkarn et al. predicted that two HIV-1 protease inhibitors (lopinavir and ritonavir) may serve as potential SARS-CoV therapeutics as they strongly bind to the Mpro active site.42 Kao et al. screened 50 240 small molecules and found 3 compounds possessing anti-SARS-CoV activity with EC50 < 10 μM.43 A similar structure-based virtual screening of 58 855 compounds found two compounds with Mpro inhibitory activity.44 Wang et al. synthesized 40 novel unsymmetrical aromatic disulfides with inhibitory activity toward Mpro.45 These compounds showed IC50 values between 0.5 and 6 μM.45 In a different study, 73 Mpro inhibitors were found using virtual screening.46 Gan et al. developed an octapeptide “AVLQSGFR” with inhibitory property against Mpro and no detectable toxicity.47 Likewise, Yang et al. developed mechanism-based irreversible inhibitors of Mpro enzyme as potent antivirals that had extremely low cellular toxicity in cell-based assays.48 However, given the fast mutation rate of SARS-CoV-2, there is a dire need to rapidly develop new and more versatile inhibitors. Consequently, special emphasis has been given to drug repurposing (i.e., new uses for existing drugs)49−52 as drugs already on the market can save both time and expenses associated with pharmacokinetic, pharmacodynamic, and toxicity studies.53,54 Many efforts have been applied computationally to identify new hits or redesign repurposed scaffolds to increase potency.55−57

Despite the considerable amount of computational and experimental data available for the Mpro protein, very few compounds have shown promising Mpro inhibition properties. To better understand the origin of their shortcomings and to systematically explore areas for improvement, a detailed structure–activity relationship (SAR) analysis was carried out using reported crystal structures and docked poses of experimentally validated Mpro inhibitors. Briefly, the present study was performed by first validating our computational protocols through docking compounds N3, 13b, 11a, and 11b into the Mpro active site and then comparing the predictions to their reported X-ray structures (Figure S1). In this respect, the superimposition of the X-ray and docked structures showed close agreement. With this procedure in place, the experimentally active compounds given in Scheme 1 were docked into the Mpro active site, and a careful analysis was performed on the ligand–protein interactions present within the S1′, S1, S2, and S3 subpockets. The active site residues surrounding each subpocket are listed in Table S0 of the Supporting Information. With this information, a set of four guiding “rules” were developed for designing potent Mpro inhibitors:

Rule 1 − The S1 subpocket should be occupied by a five-/six-membered ring containing a proton donor or acceptor (=NH or =O group) that hydrogen bonds with His163 and/or Glu166.

Rule 2 − The hydrated S1′ subpocket should feature a water bridge present between the scaffold and Thr26 and/or hydrogen/covalent bonding with the “Asn142−Gly143−Ser144−Cys145” (NGSC) motif.

Rule 3 − The S2 subpocket should be occupied by a cyclic group. Potency may be enhanced through a π−π interaction with His41 or hydrogen bonding with His41 and/or Gln189.

Rule 4 − The S3 subpocket should have its volume occupied by an appropriately sized aromatic or aliphatic group.

Adherence to the four rules and the experimental activity (IC50, Kp, or KI) of the Mpro inhibitors given in Scheme 1 are provided in Table 1. In addition, curcumin-based pyrazoline compounds from our earlier inhibition studies of human monoamine oxidase A62 and our cathepsin inhibitor from a

Figure 1. 3D structure of Mpro (PDB ID: 6L8U) from SARS-CoV-2. (a) The two monomers are shown in khaki and cyan with the catalytic site highlighted by a black box and (b) A space-filling close-up view of the catalytic site cavity (the S1, S1′, S2, and S3 subpockets are marked) and the N3 inhibitor is highlighted in gray color.
were examined as potentially repurposed S-pro inhibitors using docking and in vitro assays to assess their ability to adhere to the proposed rules and their connection to measured activity.

The crystal structure of M\(\text{pro}\) found the S1 subpocket to contain the Phe140, Leu141, Asn142, His163, Glu166, and His172 residues, Table S0. The negative charge of the pocket is mainly due to the presence of the Glu166 residue.\(^{68}\) The cocrystal structure of nirmatrelvir (FDA-authorized M\(\text{pro}\) inhibitor), N3, 13b, 11a, and 11b inhibitors bound to M\(\text{pro}\) enzyme showed the S1 subpocket to be occupied by a five-membered ring (Tables S1 and S2). This demonstrates that the S1 subpocket can readily accommodate a bulky group.\(^ {15,68}\) Moreover, the presence of a proton acceptor or donor group on the ring strengthened the interaction by forming a hydrogen bond with His163\(^ {70}\) or Glu166, respectively (Figure 2).\(^ {71}\) For example, potent M\(\text{pro}\) inhibitors designed by Jorgensen and co-workers featured the formation of hydrogen bonds with His163 or Glu166.\(^ {60}\) In addition to a five-membered ring, the crystal structure and docked poses of X77 and dipyridamole (a
The potential binding affinity of the ligand, for example, γ-lactam substituents in Mpro inhibitors, e.g., nirmatrelvir, N3, 11b, 13b, 11r, PF-00835231, and GC376 (Scheme 1) have been used successfully as a mimic for the Gln located in the P1 position of endogenous Mpro substrates. Therefore, the SAR of the S1 subpocket allowed us to propose Rule 1 in the design of Mpro inhibitors: “The S1 subpocket should be occupied by a five-/six-membered ring containing a proton donor or acceptor (−NH or −O group) that hydrogen bonds with His163 and/or Glu166.”

The S1′ subpocket is one of the most interesting and challenging sites for drug design. The analysis of multiple crystal structures suggests that the S1′ subpocket is the only hydrated region within the active site. The Mpro crystal structures bound with N3, X77, 11a, 11b, 13, 21, and PF-00835231 inhibitors have shown the presence of one or more water molecule(s) in this pocket that allows the formation of a water bridge between the ligand and protein residue Thr26 (Figures 3 and S2, and Table 2). Crystal structures of inhibitors 4, 5, and 26 (lacking a proton donor−acceptor group) did not possess the above-mentioned water bridge in these complexes despite having water molecules positioned to allow for such interactions. Recent hydrogen bonding analysis suggested that Thr26 along with Glu166 form the most stable hydrogen bonds. Moreover, the same study also identified Thr24 and Thr25 to have the highest degree of plasticity, but that plasticity was not observed in Thr26. Problematically, a lengthy or bulky substitution such as the phenyl ring of N3 in the S1′ subpocket can displace the water molecule(s) from the pocket, thereby contributing to the observed reduced activity (IC50 = 125 μM). This underlines the complexity of designing a scaffold to specifically bind within this subpocket.

Of additional interest to Mpro inhibition, the active site contains a motif composed of four residues “Asn142−Gly143−Ser144−Cys145” (NGSC) that plays an important role by providing favorable electrostatic interactions or by forming a covalent bond with the inhibitor. For example, most of the cocrystallized inhibitors, N3, nirmatrelvir, 11a, 11b, 13, X77, boceprevir, 4, 5, 14, 21, and 26, formed a hydrogen bond and/or a covalent bond with at least one residue of this NGSC motif.

### Table 1. Adherence to the “Rules” by Mpro Binding Compounds and Their Experimental Activity

| Ligand* | Activity (μM) | Rule 1 | Rule 2 | Rule 3 | Rule 4 |
|---------|--------------|--------|--------|--------|--------|
| N3      | (IC50 = 125)22 | ✔️     | ✔️     | ☒️     | ✔️     |
| Nirmatrelvir | (Ki = 0.006933)15 | ✔️     | ✔️     | ✔️     | ✔️     |
| 11b     | (IC50 = 0.94)44 | ✔️     | ✔️     | ✔️     | ✔️     |
| 11a     | (IC50 = 0.053)64 | ✔️     | ✔️     | ✔️     | ✔️     |
| X77     | (Ki = 0.057)56 | ✔️     | ✔️     | ✔️     | ✔️     |
| 13b     | (IC50 = 0.67)16 | ✔️     | ✔️     | ✔️     | ✔️     |
| Boceprevir | (IC50 = 8.0)52 | ☒️     | ✔️     | ✔️     | ✔️     |
| 4       | (IC50 = 4.02)56 | ✔️     | ✔️     | ✔️     | ✔️     |
| 5       | (IC50 = 0.14)56 | ✔️     | ✔️     | ✔️     | ✔️     |
| 14      | (IC50 = 0.128)56 | ✔️     | ✔️     | ✔️     | ✔️     |
| 21      | (IC50 = 0.018)56 | ✔️     | ✔️     | ✔️     | ✔️     |
| 26      | (IC50 = 0.170)56 | ✔️     | ✔️     | ✔️     | ✔️     |
| Dipyriddamole | (Ki = 0.04)18 | ✔️     | ✔️     | ✔️     | ✔️     |
| 11r     | (IC50 = 0.18)15 | ✔️     | ✔️     | ✔️     | ✔️     |
| 14b     | (Inactive)15 | ✗      | ✗      | ✔️     | ✔️     |
| PF-00835231 | (Ki = 0.000277)56 | ✔️     | ✔️     | ✔️     | ✔️     |
| GC376   | (IC50 = 0.15)77 | ✔️     | ✔️     | ✔️     | ✔️     |
| GB111-NH2 | (IC50 = 53.29)6 | ✗      | ✔️     | ✔️     | ✔️     |

*aChemical structures are provided in Scheme 1. bThis work.

Figure 2. Active site of Mpro enzyme cocrystallized with N3 inhibitor (PDB ID: 6LU7) highlights the importance of active site residues Glu166 and His163 for binding and activity.
especially with Gly143 (Table 2). Interestingly, residues Gly143, Ser144, and Cys145 of this NGSC motif are conserved residues, but Asn142 is not. For instance, in MERS-CoV nsp protease, a Cys residue is located at the position of Asn142 on this NSGC motif. This may suggest that Asn142 is vital in determining selectivity. Consequently, we propose that Asn142 may act as a gatekeeper residue along with His41 for the S1′ selectivity. Consequently, we propose that Asn142 may act as a gatekeeper residue along with His41 for the S1′ selectivity. Additionally, Cys145 can form a covalent bond with a ligand if there is an electrophilic warhead similar fashion to kinases.73

Table 2. Analysis of Mpro Crystal Structures to Determine the Presence of a Water Bridge between Thr25/Thr26 in the S1′ Subpocket and the Inhibitor and/or the Presence of Hydrogen/Covalent Bonding between the NGSC Motif Residues and the Inhibitor

| ligand       | PDB ID | T25/T26 | N142 | G143 | S144 | C145 |
|--------------|--------|---------|------|------|------|------|
| N3           | 6LU7   | no      | no   | yes  | no   | yes* |
| nirmatrelvir | 7TL    | no      | no   | no   | no   | yes* |
| boceprevir   | 7BRP   | yes     | yes  | yes  | yes  | yes* |
| X77          | 6W79   | yes     | no   | yes  | no   | no   |
| 11a          | 6LZE   | yes     | no   | yes  | no   | yes* |
| 11b          | 6MOK   | yes     | no   | yes  | no   | yes* |
| 13b          | 6Y2F   | no      | no   | yes  | no   | yes* |
| 4            | 7L10   | no      | no   | no   | no   | no   |
| 5            | 7L11   | no      | no   | no   | no   | no   |
| 14           | 7L12   | yes     | no   | yes  | no   | yes* |
| 21           | 7L13   | yes     | no   | yes  | no   | yes* |
| 26           | 7L14   | no      | no   | no   | no   | yes* |
| PF-00835231  | 6XHM   | yes     | no   | yes  | no   | yes* |

“Yes*” Indicates a covalent bond between Cys145 and the inhibitor.

Overall, our examination of reported crystal structures and docked poses of experimentally known Mpro inhibitors occupying the S1′ subpocket suggests that the active site water molecule(s) and NGSC motif play a pivotal role in ligand binding by providing favorable electrostatic interactions and the ability to form a covalent adduct. This allowed us to formulate Rule 2: “The hydrated S1′ subpocket should feature a water bridge present between the scaffold and Thr26 and/or hydrogen/covalent bonding with the “Asn142—Gly143—Ser144—Cys145 (NGSC) motif.”

The S2 subpocket is narrow and deeply buried in comparison to S1 and S1′, and encompasses the His41, Met49, Tyr54, and Gln189 residues. The crystallized poses indicated that, unlike the S1 subpocket, the S2 subpocket can accommodate both linear chains and cyclic rings with up to six members. While N3 featured a small noncyclical functional group bound within the S2 subpocket (Figure S1), the most potent inhibitors typically had a ring occupying this pocket instead, such as nirmatrelvir, 13b, 11a, 11b, X77, 4, 5, 14, 21, and 26 (Table S1). For example, N3 had an isobutyl group occupying the S2 subpocket that yielded a reduced activity (Table 1). Interestingly, both PF-00835231 and GC376 gave strong inhibitory activity with an experimental Kᵢ of 0.00027 μM⁶⁹ and IC₅₀ of 0.15 μM⁶⁷ respectively, despite an isobutyl substituent present in the S2 subpocket (Tables 1 and S1). Examination of each of the compounds within the active site revealed that a nitrogen atom within this peptide chain formed a hydrogen bond with the oxygen atom of Gln189. Moreover, hydrogen bonding was present from both compounds with His41. Earlier studies also suggested that hydrogen bonding interactions between an inhibitor and Gln189 may weaken with the substitution of a bulky ring, and it was identified as one of the hot spot residues.⁷¹

It has been observed that compounds 11b, 4, 5, 14, 21, and 26, which have their six-membered ring substituted with halogen groups, bind strongly to the Mpro enzyme in the S2 subpocket. Comparing the enhanced binding affinity for 11b over X77 finds that the halogen present in the former facilitates the formation of a C=π interaction with His41, whereas X77 had a t-butyl substituent present on the ring that disrupted the C=π interaction (Figure S3). Mpro nanomolar inhibitors reported by Jorgensen

Figure 3. Crystal structure of inhibitor X77 cocrytalized with Mpro (PDB ID: 6W79) features a hydrated S1′ subpocket and the formation of a water bridge [Ligand—Water—T26]. The NGSC motif conserved residues provide favorable electrostatic interactions where Asn142 and His41 may act as “gatekeeper” residues for the S1′ subpocket.

Table 2. Analysis of Mpro Crystal Structures to Determine the Presence of a Water Bridge between Thr25/Thr26 in the S1′ Subpocket and the Inhibitor and/or the Presence of Hydrogen/Covalent Bonding between the NGSC Motif Residues and the Inhibitor. Overall, our examination of reported crystal structures and docked poses of experimentally known Mpro inhibitors occupying the S1′ subpocket suggests that the active site water molecule(s) and NGSC motif play a pivotal role in ligand binding by providing favorable electrostatic interactions and the ability to form a covalent adduct. This allowed us to formulate Rule 2: “The hydrated S1′ subpocket should feature a water bridge present between the scaffold and Thr26 and/or hydrogen/covalent bonding with the “Asn142—Gly143—Ser144—Cys145 (NGSC) motif.”

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and co-workers have also shown a chlorine-substituted scaffold in the S2 subpocket. Their free energy perturbation (FEP) calculations reported gains of 2–3 kcal/mol toward the binding affinity from fluorine and chlorine substitutions at the ortho position of the benzene ring. Experimentally, those compounds were identified to be very potent (in particular compound 21) and formed a π–π interaction with His41. In addition, MM/PBSA analysis found that His41 provided a large contribution to the binding energy of the inhibitor alongside the following residues: Thr24-26, Cys145, His163, Met165, Glu166, Pro168, Gln189, and Thr190. These substituent trends observed in the S2 subpocket allowed us to formulate Rule 3: “The S2 subpocket should be occupied by a cyclic group. Potency may be enhanced through a π–π interaction with His41 or hydrogen bonding with His41 and/or Gln189.”

S3 is the fourth subpocket within the active site and is surrounded by the Met165, Leu167, Pro168, Gln189, Thr190, Ala191, and Gln192 residues (Table S0). Analysis of cocrystalized inhibitors revealed a hydrophobic S3 subpocket occupied by either a cyclic or bicyclic ring (N3, X77, 11a, 11b, and PF-00835231) or a branched alkyl chain (boceprevir and 13b) (Table S1). This suggests that various substituents may be possible in S3 when designing potent inhibitors. Analysis of interactions present between the inhibitors and residues within the S3 pocket revealed no hydrogen bonding interactions. However, inhibitors possessing an extended side chain that occupied the S3 subpocket did show enhanced activity. For example, compound 5 is ~30 times more potent than compound 4 (i.e., 4.02 vs 0.14 μM, respectively) despite having very similar scaffolds, which could be due to the extended three-carbon side chain in 5 (Figure 4a). In the same study, a difference in the functional groups interacting with the S3 subpocket showed nearly a 10-fold increase in potency for compound 21 over 26. Compound 21 showed complete occupancy of the S3 subpocket with a six-membered ring present, allowing for a tightly packed inhibitor; however, 26 had a comparatively small three-membered ring and only partially occupied S3 (Figure 4b). The potential relationship between inhibitor potency and the complete occupancy of the S3 subpocket allowed us to propose Rule 4: “The S3 subpocket should have its volume occupied by an appropriately sized aromatic or aliphatic group.”

The perceived importance of specific interactions between the reported compounds and the Mpro active site subpockets (S1, S1’, S2, and S3) raises the question “must prospective inhibitors occupy all four pockets for maximum potency?” As it turns out, it may not be necessary. For example, crystal structures of small noncovalently bound compounds that were screened against SARS-CoV-2 Mpro (PDB ID: 5R83, 5R84, 5RGZ, and 5RH3) found linearly bound compounds that occupied solely the S1 and S2 subpockets and intriguingly not the larger S1’ and S3 subpockets. Interestingly, docking various small inhibitors (Scheme 2), i.e., PX-12 (IC50 = 21.39 μM), tideglusib (IC50 = 1.55 μM), baicalein (IC50 = 0.94 μM), carmofur (IC50 = 1.82 μM), and shikonin (IC50 = 15.75 μM) into Mpro also found binding exclusively within the S1 and S2 subpockets. This may suggest importance for S1 and S2 relative to the other subpockets for small noncovalently bound inhibitors. For instance, baicalein showed well-occupied S1 and S2 subpockets in the docked pose and obeyed both Rules 1 and 3 (Table 3). A similar trend was found for other potent inhibitors such as...
Table 3. Adherence of the “Rules” by Small M<sup>pos</sup> Binding Compounds That Solely Occupy the S1 and S2 Subpockets and Their Experimental Activity

| Ligand*          | Activity (μM) | Rule 1 | Rule 2 | Rule 3 | Rule 4 |
|------------------|---------------|--------|--------|--------|--------|
| Baicalein (IC<sub>50</sub> = 0.94)<sup>23</sup> | ✓ | N/A | ✓ | N/A | N/A |
| Tideglusib (IC<sub>50</sub> = 1.55)<sup>22</sup> | X | N/A | ✓ | N/A | N/A |
| Carmofur (IC<sub>50</sub> = 1.82)<sup>24</sup> | ✓ | N/A | ✓ | N/A | N/A |
| Shikonin (IC<sub>50</sub> = 15.75)<sup>21</sup> | X | N/A | X | ✓ | N/A |
| PX-12 (IC<sub>50</sub> = 21.39)<sup>21</sup> | X | N/A | X | ✓ | N/A |

*Chemical structures provided in Scheme 2.

carmofur and tideglusib (Figure S4). However, small-molecule inhibitors that did not have the adequate length necessary to completely occupy both the S1 and S2 subpockets yielded poorer inhibitory activity, e.g., PX-12 with an IC<sub>50</sub> of 21.39 μM (Figure S4 and Table 3). Additional weak binding compounds such as shikonin also failed to fulfill Rule 1. In summary, a detailed examination of multiple small-molecule inhibitors suggests that strong interactions within the S1 and S2 subpockets (i.e., Rules 1 and 3) are crucial to designing potent M<sup>pos</sup> inhibitors and may even supersede the need to bind all four active site pockets. Weak or unfavorable interactions within the M<sup>pos</sup> sub-binding sites (S′1 and S′3) may reduce the potency of prospective inhibitors by potentially incurring an entropy penalty that is not adequately offset by enthalpic interactions.

To test our four proposed “rules” on novel compounds, a docking analysis combined with an in vitro assay study was performed on compounds available in our lab. The initial compound selected from our inventory was the cathepsin inhibitor GB111-NH<sub>2</sub> (62), as it was hypothesized that its t-shaped geometry could occupy all four M<sup>pos</sup> active site subpockets (S1, S′1, S2, and S3). However, docking analysis suggested that while this compound fulfilled Rules 2, 3, and 4, it only partially followed Rule 1 (Table 1). To clarify, GB111-NH<sub>2</sub> provided hydrogen bonding with His163 or Glu166 within the S1 pocket, but it lacked the recommended five-/six-membered ring. Given the previously discussed importance of the S1 and S2 subpockets for noncovalently bound molecules, it was little surprise that the compound yielded low experimental activity (IC<sub>50</sub> = 53.29 μM) against M<sup>pos</sup> in vitro testing (the GB111-NH<sub>2</sub> dose–response IC<sub>50</sub> curve is presented in Table S4). However, this compound did provide an active lead scaffold for future modifications; e.g., the addition of a five- or six-membered cyclic ring may improve agreement with Rule 1.

Additional small-molecule compounds from our earlier studies of curcumin-based pyrazoline inhibitors of human monoamine oxidase A, i.e., PCM-0220634, PCM-0220638, and PCM-0087495<sup>61</sup> were tested for potential inhibition of M<sup>pos</sup>. The compounds were synthesized as described in Schemes S1–S3 of the Supporting Information by following the procedure reported earlier.<sup>60,66,67</sup> Docking studies suggested that the compounds occupied different combinations of pockets, for example, S1′−S3, S1′−S2, and S2−S3; however, no inhibitors were found to simultaneously bind at S1 and S2 pockets as previously discussed for the reported small-molecule inhibitors baicalein, carmofur, and tideglusib. As such, all three compounds were found to be experimentally inactive (Table S4). It is possible that the bulky substitutions present at the phenyl ring made it difficult to fit into the S1 or the S2 subpocket. For example, in PCM-0220638 one of the phenyl rings was disubstituted with hydroxy and methoxy groups, whereas the second phenyl group was substituted with a methyl group. This lends support to our hypothesis that tight binding with the S1 and S2 active site subpockets is a major factor when designing a potent M<sup>pos</sup> inhibitor.

As a final test, both docking and experimental in vitro studies were performed for multiple Y-shaped inhibitors that should occupy no more than three of the four active site pockets: PCM-0220635, PCM-0220636, PCM-0220637, PCM-0220639, PCM-0220640, PCM-0220641, PCM-0220643, PCM-0220642, PCM-0220644, and PCM-0220646. Docking studies suggested that many of these compounds should follow Rules 1–3, but all were found to be experimentally inactive (Table S4). Given that these molecules do not fall within the linear shape, small-molecule category, binding in all four pockets may be essential for potency against M<sup>pos</sup>.

In summary, M<sup>pos</sup> in SARS-CoV-2 is an attractive drug target for COVID-19 due to its essential role in processing the polypeptides that are translated from the viral RNA. As remdesivir, nirmatrelvir, and molnupiravir are the only drugs available at present for the treatment of COVID-19, there is a dire need for novel and effective drugs against current and future SARS-CoV-2 variants. Structure–activity relationships play a crucial role in many facets of drug discovery, ranging from primary screening to lead optimization. In the current work, binding mode interactions were examined from reported crystal structures and docked poses of reported experimental M<sup>pos</sup> inhibitors. On the basis of the aforementioned SAR analysis, a set of four guiding “rules” were proposed to facilitate the creation of new inhibitors. Curcumin-based pyrazoline compounds from our earlier inhibition studies of human monoamine oxidase A were examined as potential inhibitors of M<sup>pos</sup> but were found to be experimentally inactive, which was consistent with their poor adherence to the rules. Another one of our compounds, the cathepsin inhibitor GB111-NH<sub>2</sub>, which followed Rules 2–4, but failed Rule 1, yielded weak activity (IC<sub>50</sub> = 53.29 μM) against M<sup>pos</sup> in comparison to compounds that followed all four rules, e.g., nirmatrelvir (FDA-authorized M<sup>pos</sup> inhibitor). However, this repurposed compound provides an active lead scaffold for future modifications. Overall, detailed insight into the important intermolecular interactions present within the active site was provided, and a guiding set of “rules” was created to help reduce the experimental cost and time when designing de novo compounds or repurposing existing inhibitors against SARS-CoV-2 M<sup>pos</sup>.

MATERIALS AND METHODS

Computational Details. The crystal structures of SARS-CoV-2 M<sup>pos</sup> bound with different inhibitors used in this study were N3 (6LU7),<sup>22</sup> nirmatrelvir (7TLL),<sup>32</sup> 11a (6LZE),<sup>64</sup> 11b (6MOK),<sup>64</sup> X77 (6W79),<sup>65</sup> 13b (6Y2F),<sup>55</sup> boceprevir (7BRP),<sup>67</sup> 4 (7L10),<sup>65</sup> 5 (7L11),<sup>54</sup> 14 (7L12),<sup>60</sup> 21 (7L13),<sup>60</sup> 26 (7L14),<sup>60</sup> and PF-00835231 (6XHM).<sup>69</sup> To prepare the docking calculations, the H++ server<sup>78</sup> was used to determine the protonation states of the amino acids with a careful examination of the charged groups present. Molecular docking was performed using the Autodock4.2 software to obtain the binding of ligands to M<sup>pos</sup>,<sup>79,80</sup> AutoDockTools was used to prepare the protein with all of the crystallographic water molecules removed and polar hydrogens added. Kollman charges were computed, and the atomic radii and AutoDock4 atom types were assigned. The exhaustiveness value was set to 20. The size of the grid was chosen to cover the entire active site, with a spacing of 1.00 Å. All His and Cys residues were neutrally charged; previous simulations have shown that a protonated
His172 leads to the partial collapse of the S1 pocket. All docked ligand–protein structures are provided as PDB files in the Supporting Information.

Experimental Details. Synthesis of Designed Peptide-Based Mpro Inhibitor. The SAR studies yielded an active compound, i.e., GB111-NH₂, and a series of inactive compounds, i.e., PCM series. In this study, GB111-NH₂ was synthesized using the previously reported protocol from Blum and co-workers. Compounds from the PCM series were synthesized according to Schemes S1–S3 presented in the Supporting Information.

Oxyresveratrol was purchased from Sigma-Aldrich with a purity of ≥97.0% (HPLC).

In Vitro Main Protease (Mpro) Inhibition. The Mpro activity assay procedure developed by London and Barr and at the Weizmann Institute of Science was followed in an identical fashion as previously described. Their procedure is as follows: “Compounds were seeded into assay-ready plates (Greiner 384 low volume 784900) using an Echo 555 acoustic dispenser, and DMSO was backfilled for a uniform concentration in assay plates (maximum 1%). Screening assays were conducted in duplicate at 20 μM and 50 μM. Hits of greater than 50% inhibition at 50 μM were confirmed by dose-response assays. Reagents for Mpro assay reagents were dispensed into the assay plate in 10 μL volumes for a final of 20 μL. Final reaction concentrations were 20 mM HEPEs pH = 7.3, 1 mM TCEP, 50 mM NaCl, 0.01% Tween-20, 10% glycerol, 5 mM Mpro, 375 nM fluorogenic peptide substrate ([5-FAM]-AVLQSFR-[Lys (Dabcycll)]-K-amide). Mpro was pre-incubated for 15 min at room temperature with the compound before adding substrate. The protease reaction was measured continuously in a BMG PHERAstar FS microplate reader set. The data was mapped and normalized in Gene data Screener before loading to the CDD vault.”

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpcl.2c01193.

Figures of binding modes for experimentally active compounds in Mpro; explanation of why individual compounds failed a “rule”; figure identifying water bridges present in the active site; examples of π–π interactions between inhibitors and His41; experimental IC₅₀ curves for all compounds tested; synthetic procedure for PCM series of compounds; experimental characterization of all compounds (PDF)

PDB structures for all docked SARS-CoV-2 Mpro and inhibitor complexes (ZIP)

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Notes
The authors declare no competing financial interest.

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