 doi:10.20944/preprints202204.0065.v1

Review

Advances in the Developing of SARS-CoV-2 Mpro Inhibitors

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Abstract: Since the outbreak of COVID-19, one of the strategies used to search for new drugs has been to find inhibitors of the main protease (Mpro) of virus SARS-CoV-2. Initially, previously reported inhibitors of related proteases like the main proteases of SARS-CoV and MERS-CoV were tested. Then a huge effort has been done by the scientific community to design, synthesize and test new small molecules acting as inactivators of SARS-CoV-2 Mpro. From the structure view, these compounds can be classified into two main groups: one corresponds to modified peptides displaying an adequate sequence for high affinity and a reactive warhead, and the second one is a diverse group including chemical compounds which do not have a peptide framework. Although a drug including a SARS-CoV-2 main protease has already been commercialized, denoting the importance of this field, more compounds have been demonstrated to be promising potent inhibitors as potential antiviral drugs.

Keywords: COVID-19; Main protease; Mpro; inhibitors

1. Introduction

The outbreak of COVID-19 has paralyzed the globe. As of March 22, 2022, the total confirmed cases are nearly 471 million, and the total death more than 6 million worldwide [1]. The massive vaccination campaign for COVID-19 in many countries is expected to result in herd immunity. These vaccines target the Spike protein of SARS-CoV-2 virus [2]. However, Spike protein is highly mutable as confirmed by new SARS-CoV-2 variants. The spike protein from SARS-CoV-2 shares 76 % sequence identity with the Spike protein from SARS-CoV [3]. Although booster vaccines might be developed for new variants, small molecule antivirals against less mutable targets will be more successful than a vaccine for the treatment of patients with severe symptoms and also for prevention. In general terms, administration, delivering, storage and production of small molecules are easier than vaccines. The search for new small molecules as drugs of COVID19 includes proteolytic targets in SARS-CoV-2 infection: two viral proteases called main protease (Mpro) and papain-like protease (PLpro), and three human proteases known as transmembrane protease serine 2 (TMPRSS2), cathepsin L and furin. Unlike Spike, Mpro enzyme has a highly conserved gene. The sequence identity between SARS-CoV and SARS-CoV-2 for Mpro is 96 %, much higher than Spike protein (76%) or the overall 82% genome sequence identity between both viruses.

During the replication cycle the coronavirus express two overlapping polyproteins, (pp1a and pp1b) and four structural proteins from the viral RNA. Polyproteins pp1a and pp1b liberate the mature viral proteins required for replication after being processed by two cysteine proteases coded in the viral genome: the main protease (Mpro) also known as 3-chymotrypsin-like protease (3CLpro) which carries out most of the cleavages; and papain-like protease (PLpro). Cleavage site of Mpro is between a glutamine at P1 site and a small residue at P1′, such as alanine. The P2 site is commonly occupied by a leucine residue.

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This review deals about the progress made for the search of inhibitors of SARS-CoV-2 Mpro. We will focus on the SARS-CoV-2 Mpro inhibitors which have been chemically synthesized and biologically tested.

2. Peptidyl SARS-CoV-2 Mpro inhibitors

In the search of SARS-CoV-2 Mpro inhibitors, repurposing of approved inhibitors of similar proteases was the first approach. Also, the inhibitors of SARS-CoV Mpro and MERS-CoV Mpro, both enzymes structurally similar to SARS-CoV-2 Mpro, were tested.

In April 2020, a study to identify Mpro inhibitors by combining structure-based virtual and high-throughput screening was performed. More than 10,000 compounds were assayed including approved drugs, drug candidates in clinical trials and other bioactive compounds [4]. Michael acceptor compound N3, previously reported as an inhibitor of Mpro enzymes of SARS-CoV and MERS-CoV, was identified as a time-dependent inhibitor of SARS-CoV-2 Mpro with $k_{obs}/[I]$ of 11,300 M^{-1} s^{-1} (Figure 1). Compound **N3** gave antiviral effect in SARS-CoV-2-infected Vero cells with EC_{50} value of 16.77 μM.

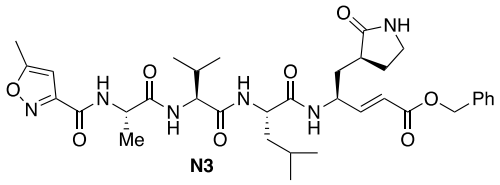

Figure 1. Chemical structure of **N3** inhibitor.

The crystal structure of SARS-CoV-2 Mpro in complex with the inhibitor was identified (2.1 Å resolution) (PDB 6LU7). The electron density showed the formation of a covalent bond between the Cβ atom of the vinyl group and the sulfur atom of Cys145 (1.8 Å C-S distance), confirming that compound **N3** acts as a Michael type inhibitor. The lactam ring of the inhibitors accommodates into the S1 site formed by the side chains of residues 140, 142, 166, 163 and 172 of protomer A of the structure, also including two ordered water molecules. At the S2 site, the isobutyl group of Leu is surrounded by residues 41, 49, 54, 165 and 187. At the S3 site, the isopropyl group of Val is solvent-exposed, and the alanine accommodates at the S4 side surrounded by residues 165, 167, 185, 192 and 189 (Figure 2) [5].

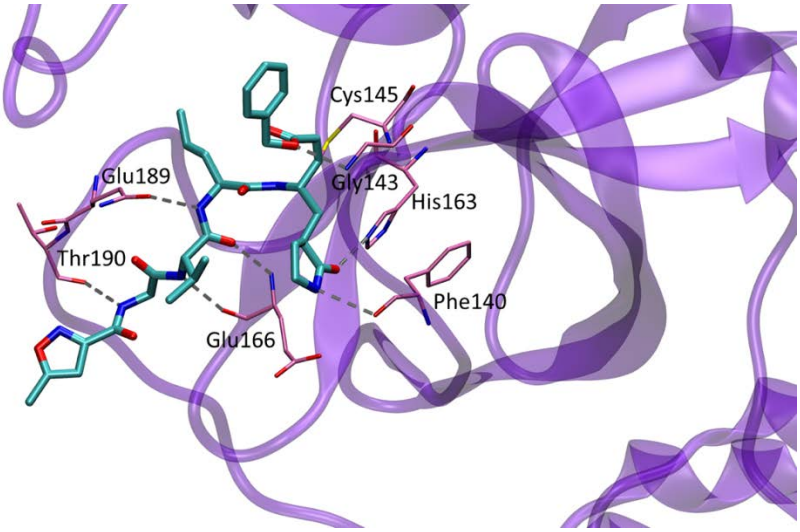

Figure 2. X-rays structure of the complex between **N3** and SARS-CoV-2 Mpro.

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Also in April 2020, R. Hilgenfeld et al. reported the ketoamides **13a** and **13b** (Figure 3) to be SARS-CoV-2 Mpro inhibitors [6]. The design of these compounds was based upon a previously reported compound active against MERS-CoV and SARS-CoV. P2-P3 amide bond in the new compounds had a pyridone to prevent cleavage by proteases hence improving half-life of the compounds in plasma. Inhibitor **13b** was active against SARS-CoV-2 Mpro (IC_{50} = 0.67 μM), and inhibited RNA replication (EC_{50} = 1.75 μM) and infected Calu-3 cells infected with SARS-CoV-2 (EC_{50} = 4-5 μM).

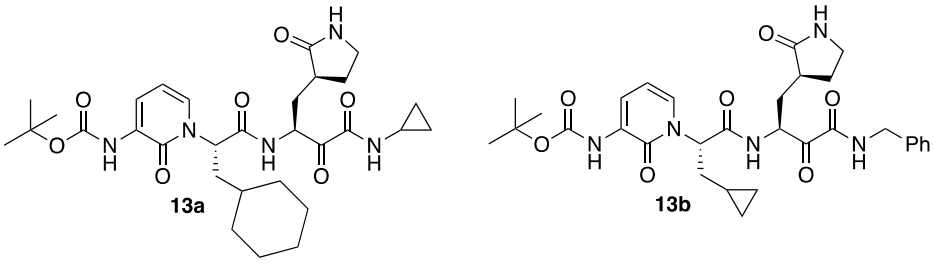

Figure 3. Inhibitors **13a** and **13b**.

The crystal structures of the complex formed between compound **13b** and SARS-CoV-2 Mpro shows a thiohemiketal resulting from the nucleophilic attack of the thiol group of Cys_{145} to the keto carbonyl group of the ketoamide moiety of **13b** (PDB 6Y2F). Thiohemiketal is stabilized in the oxyanion hole by hydrogen bonding with amides of residue His_{41}, and the amide oxygen acts as a hydrogen acceptor group from amides of Gly_{143}, Cys_{145}, and Ser_{144}. The S1 site accommodates lactam ring by hydrogen bonding with Phe_{140}, the Glu_{166} carboxylate and the carbonyl oxygen accepts a hydrogen bond from the imidazole of His_{163}. The P2 cyclopropyl methyl moiety of **13b** fits into the S2 subsite, shrunk as compared to the complex between **13a** and the SARS-CoV M_{pro} having a cyclohexyl methyl at P2 site. The carbonyl oxygen of the pyridone ring hydrogen bonds with the amide of the main chain of Glu166 whilst the nitrogen of the pyridone ring does not participate in any hydrogen bond. The Boc group does not occupy the S4 site but is slightly displaced towards Pro168 (Figure 4) [5].

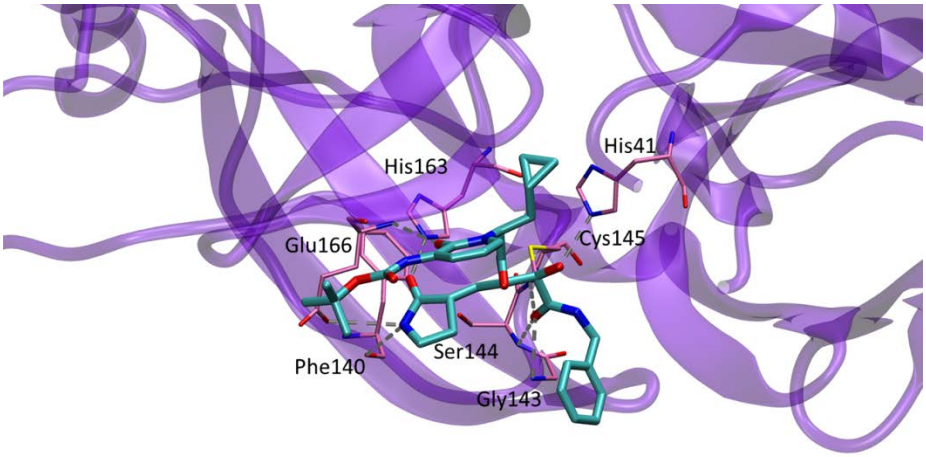

Figure 4. Detail of x-rays complex of inhibitor **13b** with SARS-CoV-2 Mpro.

In June 2020 H. Liu, H. Yang, L. Zhang, Y. Xu et al. studied the activity of compounds **11a** and **11b** as inhibitors of the protease (Figure 5). Both compounds have an aldehyde group as a warhead to be attacked by the cysteine 145 of the active center and the glutamine surrogate at P1 site. A cyclohexyl or 3-fluorophenyl ring was introduced into P2 position to test the influence of the ring. For both inhibitors, an indole group was

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introduced in P3 site to form new hydrogen bonds with S4 and improve drug-like properties. Compounds were active displaying IC_{50} values of 0.053 μM and 0.040 μM for **11a** and **11b**, respectively [7].

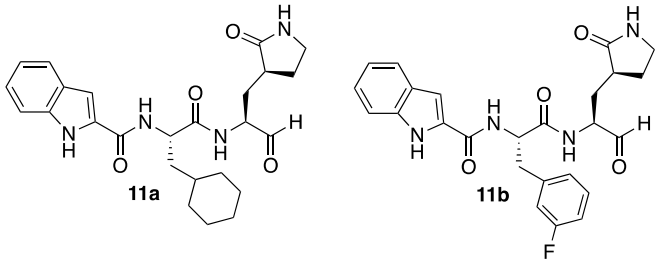

Figure 5. Inhibitors **11a** and **11b**.

The crystal structure of the complex between SARS-CoV-2 Mpro and inhibitor **11a** was determined at 1.5 Å resolution (PDB 6MOK). In the crystal, an asymmetric unit contains one molecule and the crystal belongs to the space group C2. Protomers A and B associate into a homodimer with a two-fold symmetry axis. The compound **11a** extends along the active center. The aldehyde carbon of **11a** and the sulfur of Cys145 form a C-S covalent bond (1.8 Å), and the aldehyde oxygen hydrogen bonds with the amide of Cys145. At the S1 site, the oxygen of the lactam interacts with the imidazole ring of His163 whilst the lactam NH group is hydrogen bonding with the main chain of Phe140. The cyclohexyl group at P2 interacts with Met49, Tyr54, Met165, Asp187, and Arg188 by hydrophobic interactions and stacks with the imidazole ring of His41 occupying the S2 site. The indole group of **11a** at P3 is solvent exposed and forms a hydrogen bond with Glu166 and hydrophobic interactions with Pro168 and Gln189 (Figure 6). The crystal structure of **11b** in complex with SARS-CoV-2 is very similar despite of small differences in the binding mode.

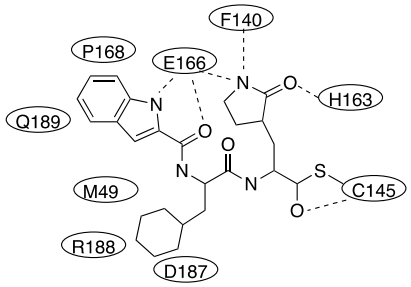

Figure 6. Scheme of the interactions in the x-rays complex of inhibitor **11a** with SARS-CoV-2 Mpro.

Inhibitors **11a** and **11b** were assayed in cell culture infected with SARS-CoV-2 virus, giving EC_{50} values of 0.53 mM and 0.72 mM, respectively. Neither compound was toxic (CC_{50} >100 mM). Both compounds showed good pharmacokinetic properties. An *in vivo* toxicity study over rats and dogs gave no obvious toxicity in either group revealing 11a as a good candidate for further clinical study.

In another study, a list of 55 known peptidyl compounds acting as inhibitors of proteases (proteasome, aspartyl, serine, cysteine and metalloproteases) were tested against SARS-CoV-2 Mpro using the FRET-based enzymatic assay [8].

Among all tested compounds, four of them (FDA-approved HCV drug boceprevir, GC-376 and calpain inhibitors II and XII in preclinical assays) (Figure 7)) had single-digit to submicromolar IC_{50} values against SARS-CoV-2 Mpro and inhibited SARS-CoV-2 viral

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replication in cell culture with low micromolar EC_{50} values. They also were low toxic with CC_{50} > 100 mM for boceprevir, GC-376 and calpain inhibitors II, and calpain inhibitor XII CC_{50} > 50 mM. The warhead of calpain inhibitor XII and boceprevir is a ketoamide and the warhead of calpain inhibitor II. Inhibitor GC-376 is a bisulfite adduct acting as a pro-drug of the active aldehyde form.

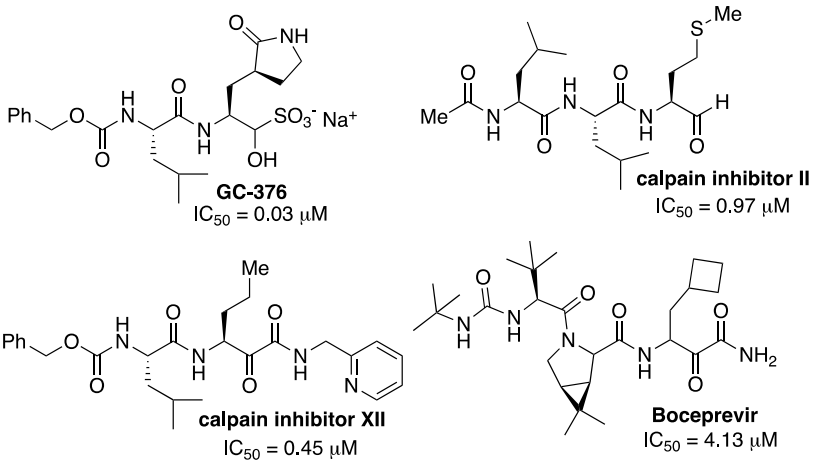

Figure 7. Inhibitors of SARS-CoV-2 Mpro enzyme.

The crystal structure of SARS-CoV-2 Mpro with inhibitor GC-376 was solved (2.15 Å resolution) revealing a hemithioketal resulting from the combination between the aldehyde warhead and Cys145 (PDB 6WTT). GC-376 mimics the substrate of Mpro by hydrogen bonding along the active site. At the S1 site, the glutamine surrogate γ-lactam ring forms hydrogen bonds with the His163 and Glu166 side chains and the main chain of Phe140. The isobutyl moiety of a leucine residue at P2 position occupies the hydrophobic site formed by His41, Met49, and Met169 (Figure 8) [5]. In other reported Mpro inhibitors, P2 position is also an isobutyl (**N3**), cyclopropyl (**13b**), cyclohexyl (**11a**), and 3-fluorophenyl (**11b**). The carbamate group in GC-376 interacts with Glu166 through a hydrogen bond and the benzyl group of the carbamate complements along the aliphatic S4 site through hydrophobic interactions.

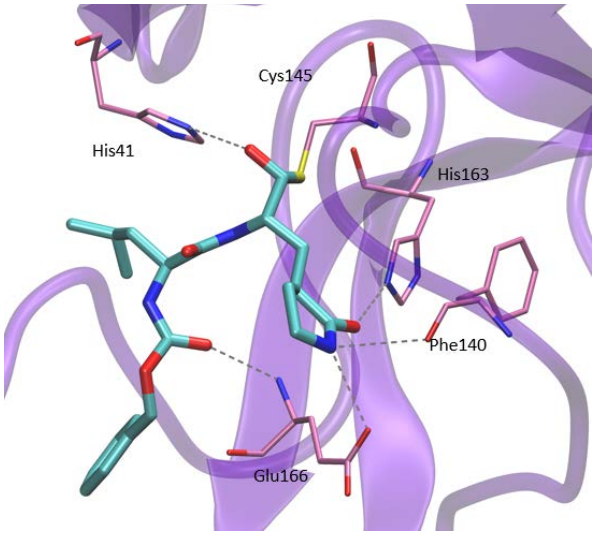

Figure 8. X-rays structure of the complex between GC-376 and SARS-CoV-2 Mpro.

More recently, GC-376 has been demonstrated to be suitable for SARS-CoV-2 therapy through a mouse model [9].

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J. Qiao et al. reported the design and synthesis of 32 new SARS-CoV-2 Mpro inhibitors [10]. The chemical structure of the new compounds had an aldehyde as a warhead, the already used glutamine surrogate at P1, a bicycloproline-containing P2 derived from either boceprevir or telaprevir, and the residue at P3 which was allowed to change (Figure 9). The IC_{50} values for *in vitro* activity ranged from 7.6 to 748.5 nM (24 compounds displayed two-digit nanomolar IC_{50} values, and three of them exhibited single-digit values). The crystal structure of the complex formed between Mpro and inhibitor MI-23 (IC_{50} = 7.6 nM) was determined (PDB 7D3I). As expected, the inhibitor fits into the active site and the carbon of the warhead aldehyde forms a covalent bond with the sulfur atom of residue Cys145. The oxygen of the aldehyde forms two hydrogen bonds with the main-chain amides of Cys145 and Gly143 in the "oxyanion hole" (Figure 9) [5]. The gamma-lactam ring at P1 forms two hydrogen bonds with His163 and Phe140 inserting deeply into the S1 pocket. The rigid P2 bicycloproline adopts the trans-exo conformation causing the bicycloproline group to point towards the S2 pocket in order to interact hydrophobically with residues Met165, Gln189, His41, Met49, Asp187 and Arg188. The 1-ethyl-3,5-difluorobenzene moiety at P3 extends along the S4 site through hydrophobic interactions with Gln189, Leu167 and Pro168. Compounds were active in cells with EC_{50} values ranging from 0.53 to 30.49 mM and showed no toxicity on cells (CC_{50} > 500 mM). Two inhibitors were assayed on rats. Two compounds showed relatively good pharmacokinetics with oral bioavailability above 10 %.

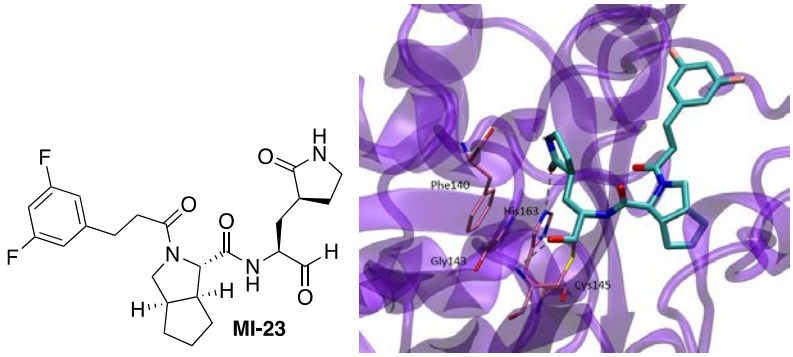

Figure 9. A) Chemical structure of Inhibitor **MI-23**. B) Detail of x-rays structure.

In September 2020, scientists from Pfizer company reported compound PF-00835231 (Figure 10) as a potent irreversible inhibitor of SARS-CoV-2 Mpro [11]. The compound had been previously identified for the treatment of SARS-CoV and given that SARS-CoV Mpro and SARS-CoV-2 Mpro share 96 % identity overall and 100% identity in the active site it, was repurposed. Compound PF-00835231 is a hydroxy ketone inhibitor. Then, the novel phosphate prodrug PF-07304814 was also described to increase the potential for the intravenous treatment of COVID-19 disease [12].

Compounds were tested *in vitro* in two cell lines (kidney and lung). For kidney cells assay, higher activity of PF-00835231 was observed if a P-glycoprotein (P-gp) transporter inhibitor was used (EC_{50} up to 0.23 μM with a concentration of 2 μM of P-gp inhibitor). No toxicity was detected. The metabolic stability studies of PF-00835231 indicated that it provides a low risk of drug-drug interactions on coadministration with other drugs. Preclinical *in vitro* and *in vivo* assays showed conversion of phosphate PF-07304814 into PF-00835231. Prodrug PF-07304814 exhibited good nonclinical safety profile, Phase 1 clinical studies are in progress.

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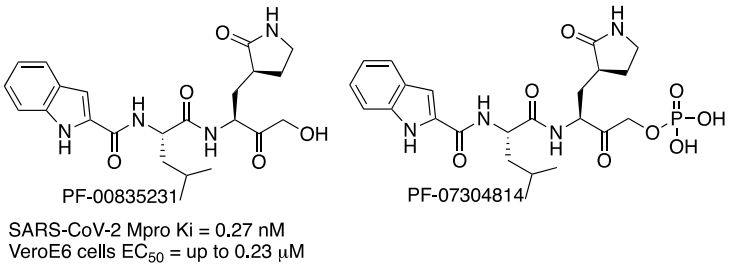

Figure 10. Pfizer inhibitors of SARS-CoV-2 Mpro enzyme.

With the objective of making an oral drug for COVID-19, scientists from Pfizer company studied new Mpro inhibitors with better oral absorption than compound PF-00835231. The warhead of previous PF-00835231 would be changed since hydroxymethyl ketone moiety is a hydrogen bond donor and it is known this type of groups correlate with poor bioavailability. Two already known warheads of cysteine proteases were considered for the new Mpro inhibitors: benzothiazol-2-yl ketone and nitrile [13]. First inhibitor of the series resulted from the substitution of the hydroxymethyl ketone warhead of PF-00835231 by a nitrile group. The resulting compound (**A**, Figure 11) showed higher rat oral bioavailability but less potency against SARS-CoV-2 Mpro and lower antiviral activity (Figure 11). If a cyclic leucine mimetic (6,6-dimethyl-3-azabicyclo[3.1.0]hexane) was introduced at P2 position and the group at P1′ acting as a warhead is a benzothiazolyl ketone then the permeability was high but the SARS-CoV-2 Mpro potency and the metabolic stability were lower (compound **B**, Figure 11). Introducing a methanesulfonamide group at P3 (compound **C**, Figure 11) instead of indole ring increased hydrogen-bonding with the Glu166 hence improving potency and antiviral activity (Figure 11). Even higher antiviral activity was obtained when a trifluoroacetamide group is introduced at P3 (compound **D**, Figure 11). Finally, best candidate (**PF-07321332**) was found when a nitrile was introduced as a warhead over this scaffold, thus giving rise to compound **PF-07321332**. This compound is a potent inhibitor of SARS-CoV-2 Mpro with improved antiviral activity (Figure 11). It inhibits SARS-CoV-2 Mpro in a reversible mode as demonstrated by competitive assays with an irreversible inhibitor. This compound was chosen as a clinical candidate based on reduced tendency to epimerization at the P1 stereocenter, ease of synthetic scale-up and enhanced solubility. This compound, named nirmatrelvir, is now commercialized as a drug for COVID-19 disease combined with protease inhibitor ritonavir and sold under brand name Paxlovid.

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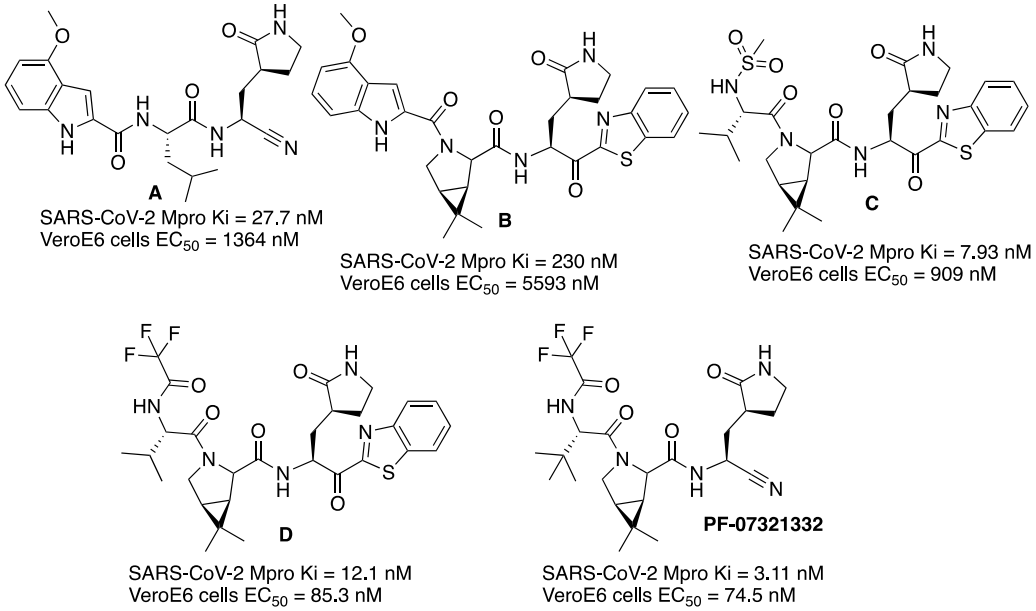

Figure 11. Pfizer inhibitors of SARS-CoV-2 Mpro enzyme.

3. Non-peptidyl SARS-CoV-2 Mpro inhibitors

In a study to identify Mpro inhibitors, more than 10,000 compounds were assayed including approved drugs, drug candidates in clinical trials and other bioactive compounds [14]. Among all tested compounds, 2-phenyl-1,2-benzoselenazol-3-one (known as ebselen) was identified as a potent inhibitor of SARS-CoV-2 Mpro (IC_{50} of 0.67 μM) (Figure 12). Ebselen is a low toxic compound which had been previously reported as having anti-inflammatory, anti-oxidant and cytoprotective properties. The compound showed the strongest antiviral effect at a concentration of 10 μM treatment in SARS-CoV-2-infected Vero cells (EC_{50} = 4.67 μM). Later Weglarz-Tomczyk et al. identified ebselen as an inhibitor of SARS-CoV-2 PLpro protease (IC_{50} = 2.26 μM) [15].

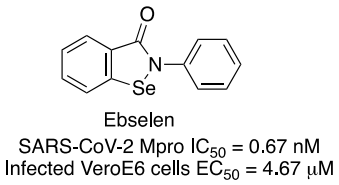

Figure 12. Chemical structure and inhibitory data of ebselen.

The mode of action of ebselen was initially studied by molecular dynamics [16]. They found two highly probable interaction sites between SARS-CoV-2 Mpro and ebselen. One is in the active site and the other one is in the region between the II and III domains, which is essential for Mpro dimerization.

Interestingly, crystal structure of the complexes formed between ebselen and its analog **MR6-31-2** with SARS-CoV-2 Mpro were solved (PDB 7BAK and 7BAL, respectively). They showed selenium atom to be bound to the sulfur atom of Cys145 but without the organic framework of ebselen (Figure 13) [5] [17]. A mechanism was suggested through attack of the sulfur to the selenium followed by hydrolysis releasing a salycinilide by-product detected by mass spectrometry (Figure 13). This mechanism has been then studied through a combined Docking and Density Functional Theory (DFT) approach [18].

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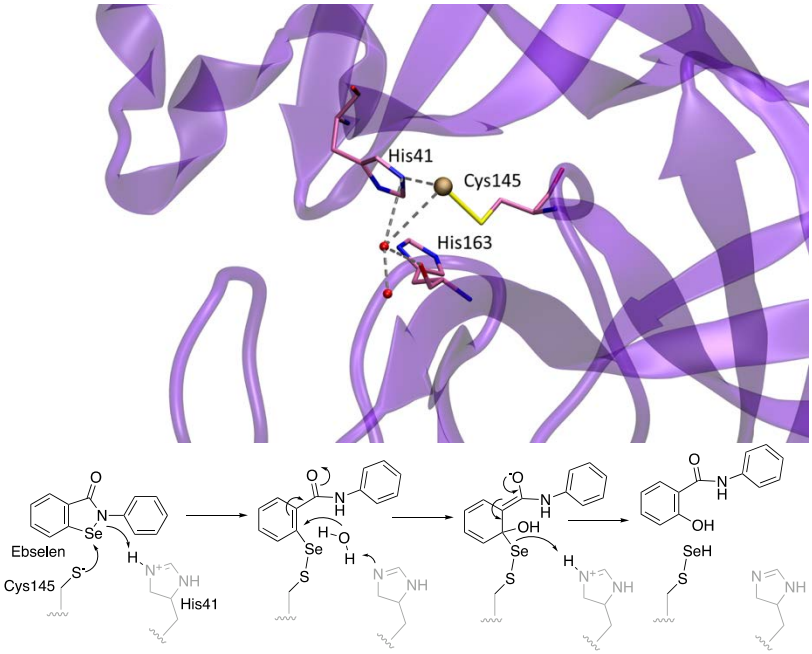

Figure 13. A) Detail of crystal structure of selenium-Mpro complex. B) Mechanism of ebselen.

K-W. Yang et al performed a FRET screening study of 36 compounds structurally derived from ebselen and ebsulfur which afforded compounds **1i** and **2k** as the most active ones (Figure 14) [19]. Interestingly both compounds displayed a furan ring as a substituent. However, these compounds were unable to effect inhibition in the presence of DTT. Also J. Wang et al studied the mechanism of action of ebselen and other five previously reported Mpro inhibitors (disulfiram, carmofur, PX-12, tideglusib, and shikonin) and concluded the inhibition is abolished or greatly reduced with the addition of reducing reagent DTT determining they are non-specific promiscuous SARS-CoV-2 main protease inhibitors [20].

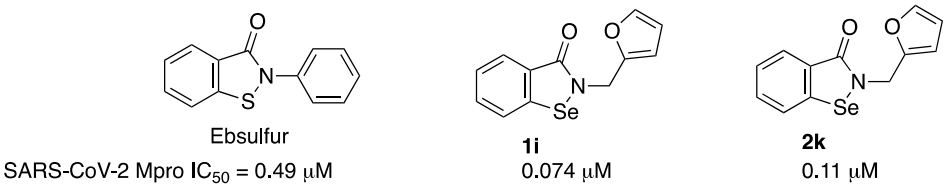

Figure 14. Ebsulfur and ebselen derivatives.

A virtual screening in ultralarge chemical libraries identified three inhibitors in a first docking study. Then, fragment elaborations gave five promising inhibitors. Finally, hit-to-lead optimization resulted in a compound with an IC_{50} of 0.39 μM for SARS-CoV-2 Mpro (Figure 15) and the crystal structure of the complex led to the design of the most potent one (IC_{50} = 0.077 μM) (Figure 15) [21]. Compounds did not show any effect on cathepsin S activity (IC_{50} > 50 μM). According to docking, the compound has a hydantoin scaffold whose carbonyl oxygens form two hydrogen bonds with Asn142 and Cys145.

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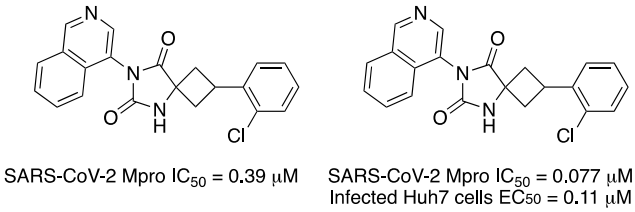

Figure 15. SARS-CoV-2 Mpro inhibitors having a hydantoin scaffold.

Two *in silico* screening studies of compounds from REAL Space or ZINC chemical compounds libraries using two structures of Mpro (PDB id: 6lu7 and PDB id: 6m0k) implemented by a fragment screening study afforded 486 compounds to be synthesized. Then, an *in vitro* protease activity assay, performed at 40 μM compound concentration, gave five compounds at 25 % inhibition level [22]. Two of them enhanced melting temperature of Mpro in a thermal shift assay as the known inhibitor GC-376 did. Then, 157 analogs of these two compounds were obtained and tested affording three compounds which were more active than parents. Finally, a second round of analogue synthesis gave dihydroquinolinone **Z222979552** as the most potent one (Figure 16). The crystal structure of Mpro in complex with compound **Z222979552** showed the compound in the active site not covalently bound to the enzyme (PDB 7P2G). It was also observed that dihydroquinolinone moiety was hydrogen bonded with Glu166, His163 and His172, and the carbonyl group of the compound hydrogen bonded the thiol group of Cys145 and the main chain of Glu166. A T-type π-stacking interaction was observed between the benzene ring of the compound and the imidazole ring of His41. Hydrophobic interactions were observed with Asn142, Met49 and Met165 residues (Figure 16). Compound **Z222979552** has antiviral activity in cells and is not toxic.

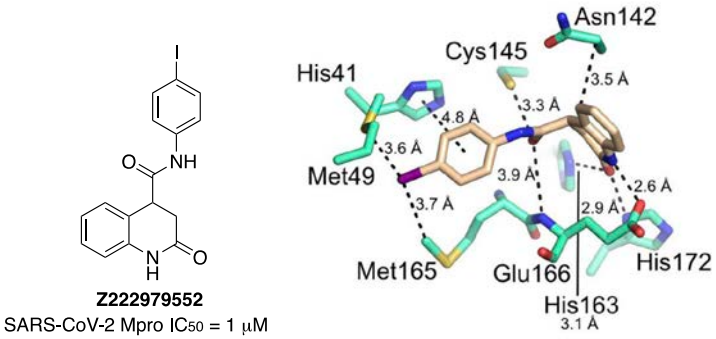

Figure 16. A) Chemical structure of compound **Z222979552**. B) Detail of crystal structure (from ref. 21).

Indole chloropyridinyl esters were prepared and tested as SARS-CoV-2 Mpro inhibitors by A K. Ghosh et al. Firstly, compound **GRL-1720** was prepared and tested [23]. It is an irreversible covalent inhibitor of SARS-CoV-2 Mpro with time-dependent inhibition kinetic parameters of k_{inact} = 2.53 ± 0.27min^{-1}, Ki = 2.15 ± 0.49 μM. The IC_{50} value for GRL-1720 after a 10 min incubation is 0.32 μM. A further study to improve indole chloropyridinyl esters analogues as SARS-CoV-2 Mpro inhibitors gave compound **7d** [24]. The mechanism of inhibition of indole chloropyridinyl ester has been studied. The inhibitor covalently modifies SARS-CoV-2 Mpro forming a thioester bond with the catalytic Cys145 and the indole carbonyl group. The catalytic dyad of Mpro, His41 and Cys145 are involved in the nucleophilic attack on the 5-chloropyridinyl ester to form a tetrahedral intermediate, which then expels the chloropyridinyl group and forms a covalent bond acylating Cys145 in the active site (Figure 17).

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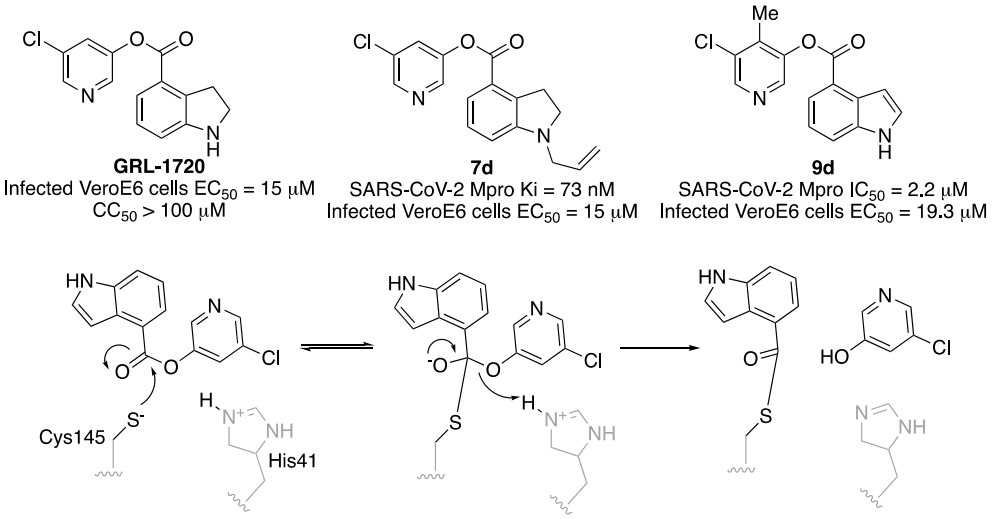

Figure 17. A) Indole chloropyridinyl esters as SARS-CoV-2 Mpro inhibitors. B) Mechanism of inhibition.

In this work, crystal structure of complex formed between inhibitor **9d** and SARS-CoV-2 Mpro (PDB 7RC0) showed sulfur atom of Cys145 to be covalently attached to the indole carbonyl group of **9d** in the S1 pocket. His41 rotates out of the way to π−π stacking with the indole ring (Figure 18) [5].

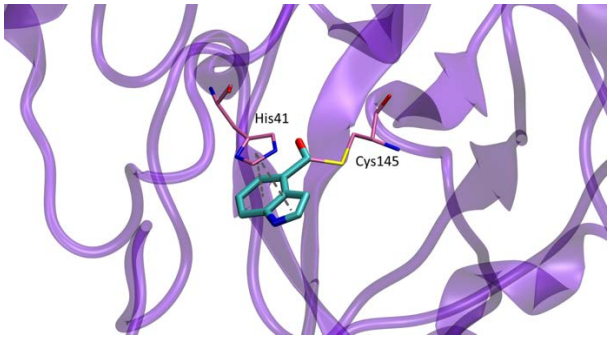

Figure 18. Detail of crystal structure of Mpro-**9d** complex.

Recently, already reported indole diketopiperazine alkaloids neoechinulin A and echinulin A have been isolated from the Red Sea-derived *Aspergillus fumigatus* MR2012 (Figure 19). Both compounds exhibited inhibitory effect against SARS-CoV-2 Mpro with IC_{50} values of 0.47 μM and 3.90 μM respectively [25].

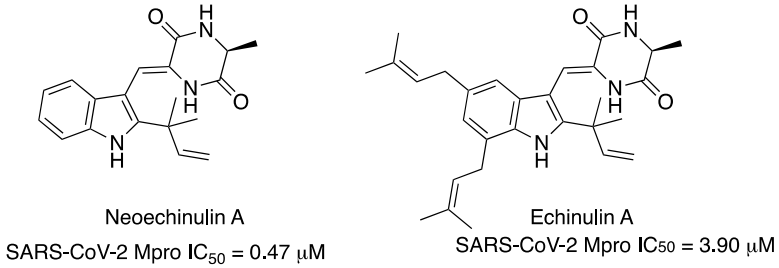

Figure 19. Marine natural products neoechinulin A and echinulin A inhibitors of SARS-CoV-2 Mpro enzyme.

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Molecular docking showed neoechinulin A binding pose establishing four hydrogen bonds between the diketopiperidine moiety and several Mpro residues from the active site: with Leu141, and with Asn142, Gly143 from S1' site, and with Glu166 from S2 site (Figure 20). Echinulin A established three hydrogen bonds via its diketopiperidine moiety and the last hydrogen bond with Glu166 was established via its indole NH. Steered molecular dynamics studies indicated that neoechinulin A has the highest binding stability inside the Mpro active site [25].

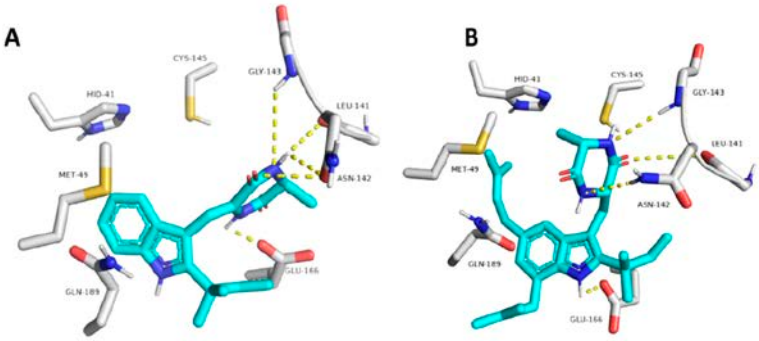

Figure 20. Binding poses of neoechinulin A and echinulin A into the active site of SARS-CoV-2 Mpro enzyme (images from ref. 24).

In a screening study of over 60 natural products, pentagalloyl glucose (PGG) and (-)-epigallocatechin-3-gallate (EGCG) were identified as inhibitors of the main protease of SARS-CoV-2 in the low micromolar range (Figure 21) [26]. The binding mechanism of these compounds takes place through hydrogen bonds and Van der Waals forces with multiple residues, including those involved in the catalytic activity, as revealed by docking.

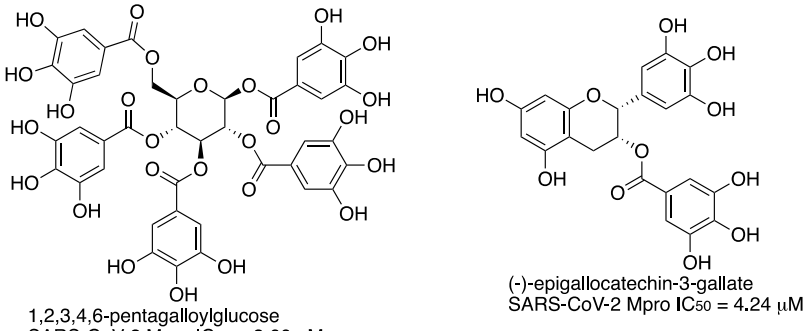

Figure 21. Natural products pentagalloyl glucose (PGG) and (-)-epigallocatechin-3-gallate (EGCG) as SARS-CoV-2 Mpro inhibitors.

A structural, computational and biochemical study identified natural product shikonin as a SARS-CoV-2 Mpro inhibitor in the low micromolar range [27]. A crystal structure of the complex formed between SARS-CoV-2 Mpro and shikonin (PDB 7CA8) revealed some interesting differences as compared to previous reported crystal structures (Figure 22) [5]. The catalytic dyad His41-Cys145 underwent dramatic conformational changes. The imidazole ring of residue His41 changed the conformation to accommodate π–π stacking interaction with the naphthoquinone ring of shikonin. Another large difference was found in a flexible loop of the protease, including Cys44 to Tyr54, Asp187 to

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Ala191, and Leu141 to Ser144, which were not located in the dimerization region and were irrelevant to crystal packing.

However, as said above, inhibition of Mpro by shikonin was abolished in the presence of DTT suggesting this compound is a promiscuous inhibitor.

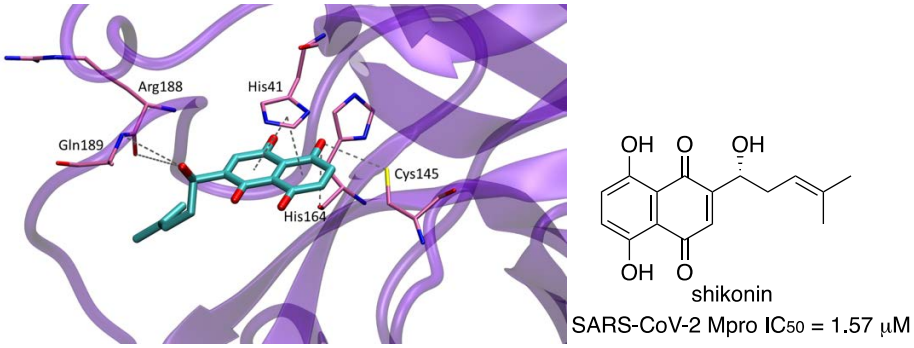

Figure 22. A) Detail of crystal structure of the complex formed between shikonin and SARS-CoV-2 Mpro. B) Chemical structure of shikonin.

9,10-Dihydrophenanthrene derivatives were tested as SARS-CoV-2 Mpro inhibitors. A Structure-Activity Relationship study resulted in the discovery of the lead compound **C1** (Figure 23). Enzyme kinetic analyses revealed **C1** dose-dependently inhibited Mpro through a mixed-inhibition manner. Molecular docking simulations elucidated the possible binding mode of **C1** at the dimer interface of the target with the hydroxymethyl group forming a hydrogen bond interaction with Gln189. Compound **C1** showed good metabolic stability [28].

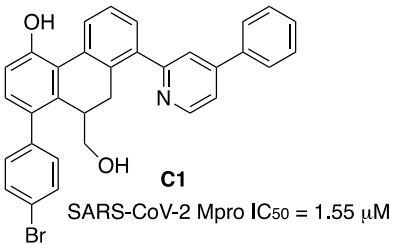

Figure 23. Dihydrophenanthrene **C1**.

An investigation of the crystal structure of the non-covalent inhibitor **X77** bound to Mpro of SARS-CoV-2 (PDB code: 6W63) showed the sulfur atom of Cys145 is positioned at 3.2 Å from the imidazole moiety suggesting that a covalent warhead could be incorporated. Thus, replacement of the imidazole with a covalent warhead appeared to be a promising strategy to improve the inhibitory potency of this noncovalent inhibitor. The resulting compound could be prepared via a four-component Ugi reaction, enabling a combinatorial approach. Among all prepared compounds, vinyl sulfone **14a** and chloroketone **16a** were the most active with one order of magnitude more potent than the original **X77** (Figure 24) [29].

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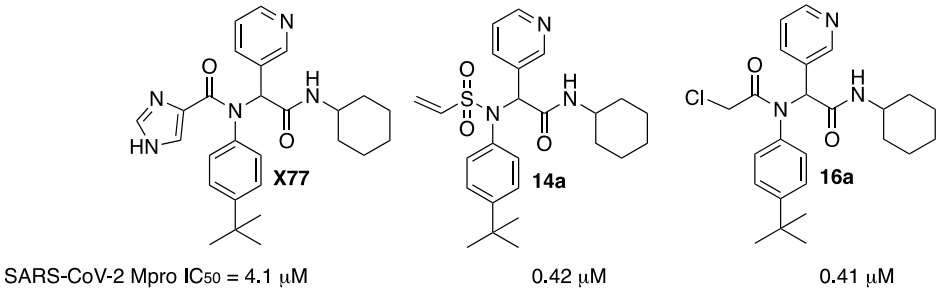

Figure 24. Inhibitors **14a** and **16a** derived from **X77**.

In summary, new molecules acting as inhibitors of the main protease (Mpro) of SARS-CoV-2 virus have been reported. Some of them show a short peptide structure usually with a glutamine surrogate at P1 site, a leucine like residue at P2 site and an electrophilic group at the carboxyl end to react with cysteine 145. Other Mpro inhibitors is formed by non-peptidic compounds being cyclic compounds or natural products. These compounds show low/sub micromolar activity in both in vitro and in vivo models and could lead to promising drug candidates for COVID-19 disease.

Author Contributions: Conceptualization, F.V.G.; writing—original draft preparation, F.V.G.; writing—review and editing, L.A., Ll. B.; S. H.; S.R.; A. F. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by Universitat Jaume I (UJI-2021-71) and SomUJIContraCovid (crowdfunding campaign). L. A. thanks Ministerio de Universidades for funding a Ph.D. fellowship (FPU19/04913). Ll. B. thanks Ministerio de Universidades for funding a Ph.D. fellowship (FPU17/06209). A. F. thanks Ministerio de Universidades for funding a Ph.D. fellowship (FPU20/03516).

Conflicts of Interest: The authors declare no conflict of interest.

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