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Structure and Function of SARS-CoV and SARS-CoV-2 Main Proteases and Their Inhibition: A Comprehensive Review

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Abstract

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) identified in 2003 infected ~8,000 people in 26 countries with 800 deaths, which was soon contained and eradicated by syndromic surveillance and enhanced quarantine. A closely related coronavirus SARS-CoV-2, the causative agent of COVID-19 identified in 2019, has been dramatically more contagious and catastrophic. It has infected and caused various flu-like symptoms of billions of people in >200 countries, including >6 million people died of or with the virus. Despite the availability of several vaccines and antiviral drugs against SARS-CoV-2, finding new therapeutics is needed because of viral evolution and a possible emerging coronavirus in the future. The main protease (M^{pro}) of these coronaviruses plays important roles in their life cycle and is essential for the viral replication. This article represents a comprehensive review of the function, structure and inhibition of SARS-CoV and -CoV-2 M^{pro}, including structure-activity relationships, protein-inhibitor interactions and clinical trial status.

Graphical Abstract

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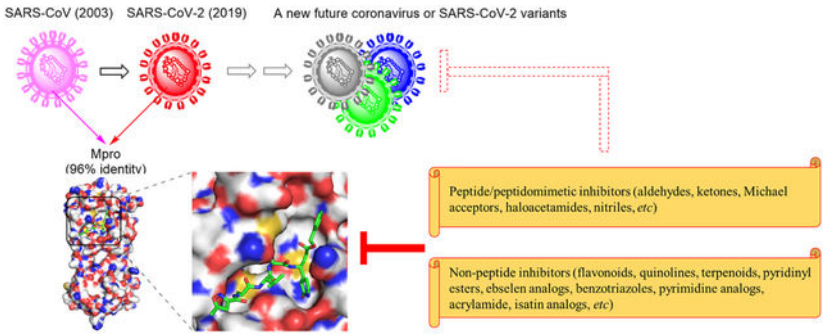
Competing interests

The authors declare that they have no competing interests.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Keywords

SARS-CoV-2; Main protease; Small-molecule inhibitor; Drug discovery

1. Introduction

Severe acute respiratory syndrome (SARS) is caused by SARS-associated coronavirus (SARS-CoV), a novel coronavirus identified in 2003. Its outbreak resulted in >8,000 cases including >800 deaths in 26 countries worldwide [1]. Without an effective treatment, SARS-CoV infection had a high mortality rate of ~10%. It was soon contained and eradicated through syndromic surveillance and enhanced quarantine. A closely related coronavirus SARS-CoV-2, the causative agent of COVID-19 discovered in Wuhan, China in December of 2019, has been dramatically more pandemic and catastrophic in the history of public health [2]. SARS-CoV-2 is highly contagious with an estimated basic reproductive number R_0 of 5.7, significantly higher than ~3 for SARS-CoV and ~1.5 for H1N1 influenza (swine flu) in 2009 [3]. It has rapidly spread to more than 200 countries worldwide and infected and caused various flu-like symptoms of billions of people worldwide, including >6 million people (mostly elderlies) died of or with the virus. In addition to upper and lower respiratory system, SARS-CoV-2 affects multiple other organs, including heart, kidney, liver and gastrointestinal and central nervous system [4]. Facing such a devastating health crisis, most countries enforced various types of “Stay at Home” orders to restrict the spread of the virus, which caused enormous negative impact to the economy. Despite the availability of several effective vaccines and antiviral drugs against SARS-CoV-2 [5], studies towards finding new targeted therapeutics are needed because of continued evolution of SARS-CoV-2 and a possible emerging coronavirus in the future.

SARS-CoV-2 is highly homologous to SARS-CoV with 82% identity in their genome sequences, particularly for several essential enzymes such as RNA-dependent RNA polymerase (RdRp, with 96% identity) and main protease (M^{pro}, with 96% identity) [6]. Studies have shown various viral and host proteins play critical roles in different stages of the life cycle of SARS-CoV-2, including the viral spike protein, RdRp, M^{pro} and papain-like protease (PL^{pro}) as well as host angiotensin-converting enzyme 2 (ACE2), cyclophilins and several other proteins [7]. Among these, M^{pro} is a promising drug target for development of antiviral agents against SARS-CoV and -CoV-2, since M^{pro} can generate 11 non-structural viral proteins [8] and is essential for replication of these viruses. This article reviews the

function, structure and inhibitors of SARS-CoV and SARS-CoV-2 M^{pro}, including protein-inhibitor interactions, structure-activity relationships, and clinical trial status. In addition, the perspectives of the antiviral drug discovery and development targeting M^{pro} of SARS-CoV-2 and closely related coronaviruses are discussed.

2. SARS-CoV, SARS-CoV-2 and other Coronavirus family members

SARS-CoV and SARS-CoV-2 belong to the Coronavirus family of RNA viruses [9], which contain four genera: α-, β-, γ-, and δ-coronavirus. α- and β-Coronavirus only infect mammals, while γ- and δ-coronavirus primarily infect birds [10]. To date, seven coronaviruses causing human diseases have been identified, including HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2 [11, 12]. Phylogenic analysis suggests all of these human coronaviruses originate from animals. HCoV-229E and HCoV-NL63 belong to α-coronavirus, while HCoV-OC43 and HCoV-HKU1 are β-coronavirus. These 4 viruses cause about one-third of common colds in humans [13] with generally mild symptoms, but they may occasionally lead to severe pneumonia and bronchiolitis in infants [14] and immunocompromised patients [15–17] or be associated with certain enteric diseases [18] and neurological disorders [19–21]. Recently identified SARS-CoV, MERS-CoV and SARS-CoV-2 are β-coronaviruses and far more pathogenic, causing serious and sometimes fatal respiratory diseases in humans.

SARS-CoV and SARS-CoV-2 are positive-sense, single-stranded RNA viruses with a genome size of 29.7 and 29.9 kb, respectively [22, 23]. Their RNA and the associated nucleocapsid proteins form a capsid [24], which is enveloped by a bilayer lipid membrane studded with the viral envelope and spike proteins (Figure 1a). The SARS-CoV and SARS-CoV-2 genomes contain an untranslated region at both the 5’- and 3’-terminus and several open reading frames (ORF) (Figure 1b). The life cycle of SARS-CoV-2 (or SARS-CoV) begins with its attachment to the host cells [9, 23, 25–27], through the interactions between its spike protein and the host cell surface ACE2 [28]. The binding triggers fusion of the viral and host cell membranes followed by endocytosis and releasing the viral RNA into the host cytoplasm, where it is translated into viral proteins. ORF1a and its frameshifted ORF1a/b give two large polyproteins, pp1a (~450 kDa) and pp1ab (~750 kDa) [8, 29], which are site-specifically cleaved by the viral proteases PL^{pro} (i.e., nsp3) and M^{pro} (nsp5) to produce 16 viral non-structure proteins (nsp), including the two proteases and RdRp (nsp12). The other RNA sequences are translated to generate viral structural proteins including the spike, envelope, membrane, nucleocapsid protein and several accessory proteins. In the meantime, RdRp is used to replicate the viral genomic RNA, which is complexed with the nucleocapsid proteins to form a capsid in the host endoplasmic reticulum-Golgi intermediate compartment (ERGIC), where a new virus particle is assembled and ready for egress to infect a new cell.

3. Structure and function of SARS-CoV and -CoV-2 M^{pro}

SARS-CoV or -CoV-2 M^{pro} (also known as $3CL^{pro}$), which cleaves the viral polyprotein and generates 11 non-structure viral proteins [8], is essential for replication of these viruses and therefore, an antiviral drug target [30]. SARS-CoV-2 M^{pro} is a 33.8-kDa protease with a high homology to that of SARS-CoV (96% sequence identity) [6] as well as other

coronaviruses (41–51% identity and 73–80% similarity). M^{pro} protein is a homodimer with two monomers oriented perpendicularly to each other (Figure 2a). Each monomer comprises three domains with the first two adopting a chymotrypsin fold for its catalytic activity. The third domain for homo-dimerization has been found to be critical to the catalytic activity [31, 32]. Similarly, the M^{pro} chymotrypsin fold is similar to other 3C-like proteases from the picornavirus family, such as rhinovirus [33, 34] causing common cold [35–39]. Figure 2 shows the structure of SARS-CoV-2 M^{pro} in complex with a representative peptidomimetic inhibitor N3 [40], which exemplifies the M^{pro}-substrate interactions (Figure 2b, c) and mechanism of catalysis that can be used for rational drug design.

M^{pro} recognizes its substrates with a consensus sequence P2P1-P1’ and hydrolyzes the amide bond between P1 and P1’, in which the P1 is always Gln, the P1’ is Ser or Ala, and the P2 is a hydrophobic Leu, Phe or Val (Figure 2d). Compound N3 occupies all of the substrate binding pockets, closely resembling an M^{pro} substrate (Figure 2b). The Cys145 -SH group undergoes a Michael addition reaction and forms a covalent bond with the acrylate group of compound N3 (Figure 2c), significantly strengthening its binding. The N3 lactam group mimics Gln (P1) and occupies the S1 pocket composed of Phe140, Leu141, Asn142, Glu166, His163 and His172. The P1 lactam forms multiple hydrogen bonds with Phe140, Glu166 and His163. The P2 Leu sidechain is located in and has hydrophobic interactions with the S2 pocket defined by His41, Met49, Tyr54, Met165 and Asp187. There is also a hydrogen bond between the amide of Leu and Gln189. The side chain of the P3 Val is open to the solvent, while the P4 Ala residue of compound N3 occupies the S4 pocket formed by Met165, Leu167, Phe185, Gln192 and Gln189. The inhibitor terminal oxazole group sitting on the edge of the pocket might have hydrophobic interactions with Pro168, Thr190 and Ala191. The benzyl ester group of the inhibitor is located in the S1’ pocket with hydrophobic interactions with Thr24 and Thr25.

Mechanistically, the –SH group of Cys145 is deprotonated by His41 and attacks the carbonyl group of the amide bond between the P1 and P1’ residues to form a thioester intermediate (Figure 2e). The protonated His41 also acts as an acid to facilitate the leaving of the P1’ amine. The ensuing hydrolysis of the thioester intermediate gives the P1 acid to complete a catalytic cycle.

4. Inhibitors of SARS-CoV and -CoV-2 M^{pro}

Due to M^{pro}’s essential roles in viral replication, a number of peptidic, peptidomimetic and non-peptidic small molecule inhibitors of M^{pro} have been designed, discovered and developed (Supporting Information Table S1). Most of these inhibitors feature an electrophilic “warhead” group, which was designed to react and covalently bind the nucleophilic -SH group of Cys145.

4.1. Peptidic and peptidomimetic inhibitors

4.1.1. Aldehyde-based inhibitors—Representative M^{pro} inhibitors with an aldehyde “warhead” are shown in Figure 3. Peptidomimetic aldehydes, including compound **11a**, were reported as covalent inhibitors of SARS-CoV-2 M^{pro} [41]. **11a** demonstrated potent enzymatic activity with an IC_{50} value of 53 nM and cellular antiviral EC_{50} value of 530

nM. Its binding structure in SARS-CoV-2 M^{pro} as well as in vivo pharmacokinetics and toxicities were also studied. Compound **18p** with a phenyl group at the P2 position instead of cyclohexyl group in **11a** also retained the activities [42].

Compound GC373 and its bisulfite adduct prodrug GC376, initially used to treat feline coronavirus, were found to inhibit M^{pro} [43] with submicromolar IC_{50} values. These compounds showed strong anti-SARS-CoV-2 activity in cells with EC_{50} values of 1.5 and 0.92 μM. GC376 was also reported by other groups [44–47] and showed broad-spectrum activity against coronaviruses, including SARS-CoV [48]. However, it showed poor pharmacokinetics and limited in vivo anti-SARS-CoV-2 activity in mice [49, 50]. Modification of GC376 yielded inhibitors with moderately improved enzymatic and cellular activities [51]. Interestingly, deuterated GC-376 showed significantly enhanced potencies in SARS-CoV-2 infected cells and mice [52], despite its similar enzymatic activity to the parent inhibitor. X-ray crystallographic studies also indicated the deuterated GC-376 binds to SARS-CoV-2 M^{pro} similarly. Compared with GC373/GC376, compounds with different capping groups at the P3 position [53–60], such as compounds **6e** [53] and **2a** [54] also showed similar or better biochemical and cellular antiviral activities. Calpeptin with a *n*-butyl P1 sidechain showed modest activity against SARS-CoV-2 M^{pro} (IC_{50} = 10.69 μM) [47], but it exhibited a potent antiviral EC_{50} of 72 nM in Vero cells [61], presumably due to off-target effects. MPI8/TG-0205221 was found to exhibit dual inhibition of SARS-CoV M^{pro} (K_i = 53 nM) and human cathepsin L (which is also a cysteine protease critical to the virus entry), with good selectivity over other cathepsins [62, 63]. It showed low-nM antiviral activities together with stabilities in mouse, rat and human plasma [62]. However, despite its increased enzymatic activity, analogous compound MPI3 with smaller hydrophobic P2 and P3 sidechains had no antiviral activity up to 10 μM, possibly due to cellular stability issues [64]. Other similar aldehyde inhibitors were reported [47, 48, 64–74].

M^{pro} inhibitor MI-09 with an azabicyclo[3.1.0]hexane P2 moiety is one of the most potent inhibitors with an IC_{50} of 15.2 nM. It showed potent anti-SARS-CoV-2 activities in cell-based assays (EC_{50} = 0.86 μM) and in a mouse model. MI-09 also possesses good pharmacokinetics [70], e.g., $T_{1/2}$ = 4.53 h. Structurally similar compounds, such as UAWJ9–36-3, were also reported [71, 75]. In another study, a variety of proline derivatives at the P2 were explored, which led to the finding of compound **12** with an IC_{50} of 5 nM against SARS-CoV-2 M^{pro} and an antiviral EC_{50} of 5.3 μM, while it showed a moderate cytotoxicity (CC_{50} of 28.4 μM) [76].

4.1.2. Ketone-based inhibitors—Although aldehyde-based inhibitors of M^{pro} have potent enzyme activities, the aldehyde group is chemically reactive and often associated with off-target effects and undesired toxicities. Less electrophilic ketone has been explored as the "warhead" group of M^{pro} inhibitors (Figure 4).

Benzothiazolyl ketone **5h**/YH-53 was found to be a potent inhibitor of SARS-CoV M^{pro} (K_i = 6 nM) [77–80] and SARS-CoV-2 M^{pro} (K_i = 18 nM) with cellular antiviral activities [81]. In addition, it had no cytotoxicity and showed favorable in vivo pharmacokinetics except for a low oral bioavailability [82]. Structurally similar PF-00835231 with a hydroxylmethyl ketone "warhead" retained potent biochemical and antiviral activities (IC_{50} = 6.9 nM and

EC_{50} = 231 nM) with more favorable drug properties [83–86]. Phase I clinical trials (NCT04627532 and NCT04535167) of its phosphate prodrug PF-07304814 have been completed, showing good safety profiles [84, 85]. Moreover, in a comparison study, the hydroxymethyl ketone-based inhibitor was found to exhibit more biochemical activity against SARS-CoV-2 M^{pro} than does structurally similar, nitrile-based nirmatrelvir [87].

Heteroaromatic and aliphatic α-acyloxymethyl ketone warheads were found in a series of SARS-CoV-2 M^{pro} inhibitors, such as compound **15l** showing biochemical IC_{50} of 19 nM and cellular antiviral EC_{50} of 300 nM without overt cytotoxicity [88]. A similar phenyl α-acyloxymethylketone compound was also reported with weak activities as well as poor protease selectivity [89].

α-Ketoamide compounds were reported to be M^{pro} inhibitors [32, 90]. Compound **11r** exhibited an IC_{50} of 0.71 μM against SARS-CoV M^{pro} and inhibited the viral replication in Vero cells with an EC_{50} of 2.1 μM [90]. Incorporation of P3-P2 amide bond of **11r** into a pyridone ring led to the discovery of **13b** with good drug-like properties, although its enzymatic and cellular activities were slightly compromised [32]. A subsequent study indicated that one diastereomer (**13b-K**) of **13b** with a *S*-P2 moiety is a more potent inhibitor with an IC_{50} of 0.12 μM, but the corresponding *R*-enantiomer is almost inactive (IC_{50} > 5 μM) [91]. **13b-K** had more potent anti-SARS-CoV-2 activity with EC_{50}s of 0.84–3.4 μM. Calpain inhibitor XII with a *n*-propyl P1 sidechain showed potent inhibition of SARS-CoV-2 M^{pro} as well as a broad spectrum of anti-coronavirus activities including SARS-CoV and -CoV-2 [47, 48]. Anti-hepatitis C virus drug boceprevir with a cyclobutylmethyl P1 moiety was found to be an inhibitor of SARS-CoV-2 M^{pro} with IC_{50} values ranging from 0.95 μM to 8.0 μM by several research groups [44, 47, 92–96]. It also exhibited broad anti-coronavirus activities including SARS-CoV-2 (EC_{50} = 1.3–19.6 μM) [44, 47, 48, 92]. With a 5-membered lactam at the P1 position that mimics Gln, structurally similar ML1000 showed significantly increased potencies (IC_{50} = 12 nM and EC_{50} = 100 nM) [97]. However, narlaprevir with a *n*-butyl P1 showed similar activities to boceprevir [47, 92, 95, 98]. SY110, obtained from compound screening followed by structural modification, exhibited strong inhibition against SARS-CoV-2 M^{pro} with an IC_{50} of 14.4 nM and broad anti-coronavirus activity including different variants of SARS-CoV-2 and SARS-CoV with EC_{50}s in sub-μM to low μM range [99]. With favorable pharmacokinetic properties and safety profiles, oral administration of SY110 significantly protected mice infected with SARS-CoV-2 (Omicron strain).

Compound Y180, having a methyl ketone “warhead” group, was reported to be a potent SARS-CoV-2 M^{pro} inhibitor (IC_{50} = 8.1 nM) with advanced preclinical studies. It exhibited excellent antiviral activities against wild-type and mutant SARS-CoV-2 with EC_{50}s of 11.4–34.4 nM [100]. With good oral bioavailability (92.9% in mice), pharmacokinetics (e.g., $T_{1/2}$ = 1.42 h) and no overt toxicities, Y180 showed strong anti-SRAS-CoV-2 activities in several animal models. Other M^{pro} inhibitors with a phthalhydrazido, or trifluoromethyl-ketone were also reported with low or untested antiviral activities [77, 101–103].

4.1.3. α, β-Unsaturated esters and related Michael acceptors—

α, β-Unsaturated ester, amide and related groups can covalently bind Cys145 through a Michael addition

reaction and is therefore a good “warhead” group for cysteine proteases. Representative inhibitors are shown in Figure 5. Rupintrivir (AG7088), a potent inhibitor of rhinovirus 3CL^{pro}, was found to have negligible inhibitory activities against M^{pro} of SARS-CoV and -CoV-2 (IC_{50} >= 68 μM) [30, 104–106], but it strongly inhibited replication of SARS-CoV-2 with an EC_{50} of 1.87 μM [107]. Compound **18c** with a cinnamoyl P3 moiety showed good inhibitory activities against SARS-CoV M^{pro} (IC_{50} = 1 μM) as well as cellular viral replication (EC_{50} = 0.18 μM) without overt toxicity [105].

Structure-based drug design led to the discovery of SARS-CoV M^{pro} inhibitor N3 (Figure 2b) with a K_i of 9.0 μM [108]. It is also an inhibitor against SARS-CoV-2 M^{pro} as well as the virus replication with an EC_{50} of 16.77 μM [40]. TG-0203770, with a 1-(*tert*-butoxy)ethyl moiety at P3, is a potent inhibitor of SARS-CoV and -CoV-2 M^{pro} (K_i = 58 and 151 nM) [109] with a strong cellular anti-SARS-CoV-2 EC_{50} of 2.88 μM [45]. SM141 with a benzyl group at the P2 and P3 showed dual inhibition of SARS-CoV-2 M^{pro} and human Cathepsin L with IC_{50} of 0.9 μM and 60 nM, respectively [110]. It potently inhibited cellular replication of SARS-CoV-2 with an EC_{50} of 8.2 nM without cytotoxicity. It can significantly reduce viral loads and prolong animal survivals in SARS-CoV-2-infected mice. Other α, β-unsaturated ester-based and related inhibitors of SARS-CoV and -CoV-2 M^{pro} were also reported [109, 111–114], among which SPR39 with a vinyl methyl ketone Michael acceptor showed strong SARS-CoV-2 M^{pro} inhibition with a K_i of 0.252 μM and an antiviral EC_{50} of 1.5 μM [114].

Similarly, acrylamide or vinyl sulfone could undergo a Michael addition reaction and be a potential warhead. Acrylamide compound MPI80 was reported to potently inhibit SARS-CoV-2 M^{pro} with an IC_{50} of 34 nM. It blocked cellular viral replication with an EC_{50} of 0.70 μM [115]. Several other acrylamide- [116] or vinyl sulfone-based inhibitors of SARS-CoV-2 M^{pro} [117, 118] were also reported with low or untested antiviral activities.

4.1.4. Haloacetyl-based inhibitors—

Compound screening identified a chloroacetamide compound JCP400 (Figure 6) to be an inhibitor of SARS-CoV-2 M^{pro} with an IC_{50} of 1.74 μM as well as moderate antiviral activities [89]. But it had similar activities against other proteases, such as cathepsins L and B, showing a poor selectivity. Jun9–62-2R with a dichloroacetamide group was developed as a covalent inhibitor of SARS-CoV and -CoV-2 M^{pro}, showing biochemical IC_{50} and cellular antiviral EC_{50} in sub- to low-μM range [119]. Azapeptidic compounds bearing a mono- or di-chloroacetamide, such as MPI89, were reported to be a potent SARS-CoV-2 M^{pro} inhibitor [115]. MPI89 exhibited potent antiviral activities with low cytotoxicity, but it had a short half-life in plasma (~ 20 min). Compound **29** was discovered as a covalent inhibitor of SARS-CoV-2 M^{pro} with an IC_{50} of 1.72 μM [120], but it showed more potent inhibitory activity against SARS-CoV-2 PL^{pro} (IC_{50} = 0.67 μM). Compound **29** was found to inhibit replication of a variety of SARS-CoV-2 strains in Vero cells with EC_{50}s of 0.32–1.37 μM. Other haloacetyl-based inhibitors were also reported [117, 121–124], among which several compounds showed potent biochemical inhibition, but none of them were tested in cells or animals [117, 122, 123].

4.1.5. Nitrile-based inhibitors—

Modifications of hydroxylmethyl ketone-based inhibitor PF-00835231 (Figure 4) led to the discovery of M^{pro} inhibitor nirmatrelvir

(initially coded as PF-07321332) with a nitrile “warhead” (Figure 6). nirmatrelvir showed a highly potent activity (K_i = 3.11 nM) against SARS-CoV-2 M^{pro}. While it is less active than PF-00835231 (K_i = 0.27 nM), nirmatrelvir exhibited ~3× more cellular antivirus activities [86]. Oral administration of nirmatrelvir significantly reduced the viral loads in SARS-CoV-2 infected mice and protected them from weight losses [86]. Furthermore, immunohistochemical analysis also revealed it significantly alleviated virus-caused lung damages in a dose-dependent manner. Nirmatrelvir exhibited a good safety profile with a high selectivity over a broad panel of human proteins. It is also negative in the genetic toxicity studies and rat micronucleus assay. Furthermore, the embryo-fetal, fertility and early embryonic development studies indicated nirmatrelvir is a safe drug in animal models [125]. Other nirmatrelvir analogs with the same P1, P2 and P3 were also reported [126].

Another nitrile compound **18b** (Figure 6) with an indole P3 moiety showed potent biochemical activity and strong antiviral activity against SARS-CoV-2, together with a good selectivity over human cysteine proteases [127]. In addition, nitrile-based peptidomimetic compound Cbz-AVLQ-CN, designed based on the autocleavage tetrapeptide sequence of SARS-CoV M^{pro}, was found to have an IC_{50} of 4.6 μM as well as a broad inhibitory activities against other coronavirus M^{pro} with IC_{50}s of 1.3–4.6 μM [128, 129]. Compound screening followed by medicinal chemistry studies identified azanitrile compound Gü3619 (Figure 6) as a potent irreversible covalent inhibitor of SARS-CoV-2 M^{pro} with an IC_{50} of 37.8 nM, but it also potently inhibited human cathepsins L and B [130].

4.1.6. Other miscellaneous compounds—

Epoxy ketone compound WRR183 (Figure 7) was found to inhibit SARS-CoV M^{pro} with a K_i of 2.2 μM and the virus replication with an EC_{50} of 12 μM [131]. Its electrophilic epoxy β-carbon atom forms a thioether bond with M^{pro} Cys145. Several substrate-based oligomeric peptides or peptidomimetic compounds were reported to be inhibitors of SAR-CoV and/or -CoV-2 M^{pro} with sub-μM activities [132–140].

Virtual screening followed by medicinal chemistry led to the discovery of non-covalent inhibitor ML188 (Figure 7) with IC_{50}s in the low μM range against SARS-CoV and -CoV-2 M^{pro} [141, 142]. In the cell-based assay, ML188 exhibited anti-SARS-CoV activity with an EC_{50} value of 13 μM. Modification of ML188 yielded more potent inhibitor 23R (Figure 7) [143].

Anti-HIV drug atazanavir, an inhibitor of HIV-1 (aspartic) protease, was found to inhibit SARS-CoV-2 M^{pro} as well as cellular viral replication in the sub-μM to μM range [144, 145]. It also exhibited significant anti-SARS-CoV-2 activity in mice [145]. Cobicistat, an inhibitor of human cytochrome P450 and an adjuvant drug for HIV treatment, was reported to be a SARS-CoV-2 M^{pro} inhibitor with an IC_{50} of 6.7 μM [107, 146]. However, inhibition of SARS-CoV-2 M^{pro} by these two drugs were not confirmed by other researchers [65, 147]. In addition, immune-modulating polypeptide drug glatiramer acetate was also identified to be a weak SARS-CoV-2 M^{pro} inhibitor with mild antiviral activity [148].

4.2. Non-peptidic small molecule inhibitors

Non-peptidic inhibitors of SARS-CoV and -CoV-2 M^{pro} have been discovered and developed, with the majority initially identified from compound screening including virtual screening.

4.2.1. Flavonoids—Natural flavonoid compound baicalin (Figure 8) was identified as an inhibitor of SARS-CoV-2 M^{pro} with an IC_{50} of 6.41 μM, which inhibited replication of SARS-CoV-2 in cells (EC_{50} = 27.87 μM) [149, 150]. Baicalein, the parent compound of baicalin, exerted improved biochemical and cellular activities against SARS-CoV and -CoV-2 [149–151]. Analogs with more hydroxyl groups in the 2-phenyl substituent of baicalein, such as myricetin, retained the biochemical activity [152, 153]. Interestingly, myricetin was found to be oxidized by O_2 to become a quinone and covalently bind to Cys145 of SARS-CoV-2 M^{pro} (Figure 18g, h) [152]. However, flavonoid compounds without the 2-phenyl substitution, such as esculetin-4-carboxylic acid ethyl ester, is a weak SARS-CoV M^{pro} inhibitor [154]. Other flavonoids and related analogs have also been reported to inhibit M^{pro} with low-μM IC_{50} values [151, 155–171], while their antiviral activities were not disclosed.

4.2.2. Quinoline analogs—Quinoline compound MAT-POS-e194df51-1 (Figure 9) was identified in an X-ray-based fragment screening campaign to be a potent SARS-CoV-2 M^{pro} inhibitor (IC_{50} = 36.8 nM), which exhibited potent anti-SARS-CoV-2 activities with EC_{50} as low as 63.8 nM [172]. It had acceptable in vivo pharmacokinetic and toxicities (e.g., $T_{1/2}$ = 1.4 h in rats) with oral bioavailability (18% in rats). Quinoline compound DA-3003-1 showed an inhibitory IC_{50} of 2.63 μM against SARS-CoV-2 M^{pro} and an EC_{50} of 4.47 μM in the SARS-CoV-2 cytopathic effect (CPE) assay, but it is cytotoxic (CC_{50} = 7.74 μM) [173]. Compound **19** was discovered from virtual screening followed by structure-based optimization, having an IC_{50} of 77 nM against SARS-CoV-2 M^{pro} as well as antiviral EC_{50} values as low as 77 nM [174]. Compound **C7**, from structure-based drug development based on baicalein (Figure 8), exhibited a potent activity against SARS-CoV-2 M^{pro} (IC_{50} = 85 nM) and cellular viral replication (EC_{50} = 1.10 μM) [175].

Several quinolone or related drugs were reported to inhibit M^{pro} of SARS-CoV and -CoV-2. Anti-hepatitis C virus (HCV) drug simeprevir, an inhibitor of HCV NS3/4A (serine) protease, inhibited SARS-CoV-2 M^{pro} with IC_{50} of 2.46–13.74 μM [47, 176, 177], while it also showed comparable activities against SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) (IC_{50} = 5.5 μM) [177]. Simeprevir inhibited replication of SARS-CoV-2 in Vero cells with an EC_{50} of 1.40 μM [176]. Nelfinavir, a HIV-1 protease inhibitor, was reported to only partially inhibit SARS-CoV M^{pro}. It can inhibit cellular replication of SARS-CoV and -CoV-2 with EC_{50} values of 0.048–3.3 μM by several groups [107, 178–180]. However, nelfinavir was found to not inhibit M^{pro} by another laboratory [65]. Also, nelfinavir was unable to inhibit SARS-CoV-2 replication in hamsters [181]. Pelitinib, an anticancer drug inhibiting human epidermal growth factor receptor (EGFR), was found to bind to an allosteric site of SARS-CoV-2 M^{pro}. Although it inhibited proliferation of SARS-CoV-2 in Vero E6 cells with an EC_{50} of 1.25 μM, its biochemical activity against M^{pro} was not disclosed [61].

Compound screening or rational drug design led to discovery and development of other quinoline analogs [65, 182–194], with the best compounds showing strong inhibition of M^{pro} [185, 186, 188, 193]. But their cellular anti-SARS-CoV-2 activities were not evaluated or weak.

4.2.3. Terpenoids—Bardoxolone methyl (Figure 10) was identified as a SARS-CoV-2 M^{pro} covalent inhibitor through screening of compounds bearing an electrophilic group [195]. With a moderate enzyme activity (IC_{50} = 5.81 μM), it showed potent cellular antiviral activity (EC_{50} = 0.29 μM). A cell-based M^{pro} inhibitor screening yielded hydroxyprogesterone (cellular IC_{50} of 2.47 μM), which blocked replication of SAR-CoV-2 in Vero cells with an EC_{50} of 2.77 μM [176]. The *S*-enantiomer of phloroglucinol terpenoid **3** was identified to be an inhibitor of SARS-CoV-2 M^{pro} from virtual screening with an IC_{50} of 7.5 μM and antiviral EC_{50} of 4.5 μM, while its *R*-isomer was less active [196]. Several other terpenoid compounds, including betulinic acid, were found to inhibit SARS-CoV and -CoV-2 M^{pro} with micromolar IC_{50} values [197–203].

4.2.4. Pyridinyl ester and related compounds—Activated esters that could covalently bind to Cys145, including pyridinyl esters [45, 81, 130, 204–207], benzotriazole esters [208], and their analogs [209–212], were explored as SARS-CoV and -CoV-2 M^{pro} inhibitors. Pyridinyl ester compound GRL-0920 (Figure 11) was found to be a potent inhibitor of SARS-CoV M^{pro} with an IC_{50} of 30 nM, which blocked the cellular viral replication with an EC_{50} of 6.9 μM [204]. It also showed comparable activities against SARS-CoV-2 M^{pro} and replication [205, 206]. Other analogs of GRL-0920 were developed with similar or reduced activities [81, 205, 206]. Mechanistically, the -SH group of Cys145 nucleophilically attacks and hydrolyzes the activated ester, with the 5-chloropyridin-3-ol being a good leaving group. The thioester product has been confirmed by X-ray crystallography and mass spectrometry studies [205, 206]. Several other pyridyl esters or their analogs were also found to be M^{pro} inhibitors [45, 130, 207, 212, 213], including WNN2048-F004 (IC_{50} = 103.1 nM). However, it only showed modest cellular antiviral activity against SARS-CoV-2.

A series of thioesters were reported, including compound **3w** with IC_{50}s of 61.3 and 11.4 nM against SARS-CoV and -CoV-2 M^{pro} [214]. It showed an EC_{50} of 0.11 μM against the replication of SARS-CoV-2 virus without cytotoxicity up to 10 μM. In addition, benzotriazole esters were reported as irreversible SARS-CoV M^{pro} inhibitors [208]. The most potent compound **8** (Figure 11) showed potent inhibition of M^{pro} with a K_i of 7.5 nM, while no cellular antiviral activities were disclosed. X-ray crystallography and mechanistic studies revealed that these inhibitors acylate the active site Cys145 with their benzotriazole being as a leaving group [215]. Moreover, dithiocarbamate compound **1**, identified from high-throughput screening (HTS), potently inhibited M^{pro} of SAR-CoV-2 (IC_{50} = 21 nM), SARS-CoV (IC_{50} = 383 nM) and other coronaviruses. It covalently modifies Cys145 to form a dithiocarbamate adduct. Compound **1** was found to inhibit cellular proliferation of SARS-CoV-2 with an EC_{50} of 1.06 μM [216].

4.2.5. Ebselen analogs—Through high-throughput screening, selenium-containing compound ebselen (Figure 11) was found to be an M^{pro} inhibitor with an IC_{50} of 0.67 μM,

which inhibited cellular replication of SARS-CoV-2 with an EC_{50} value of 4.67 μM [40]. It was later reported to be a nonspecific inhibitor [65, 217, 218]. Modification of ebselen has yielded several more potent inhibitors of SARS-CoV-2 M^{pro} [219–227], including MR6–18-4 with an IC_{50} of 0.35 μM as well as cellular anti-SARS-CoV-2 EC_{50} of 3.74 μM. An X-ray crystallographic study suggested that ebselen (and its analog) covalently modifies the Cys145 -SH group of SARS-CoV-2 M^{pro} by forming a S-Se bond, while other atoms of ebselen cannot be found in the structure [220].

4.2.6. Benzotriazole-based inhibitors—Benzotriazole compound ML300 (Figure 12) was identified as a SARS-CoV-2 M^{pro} inhibitor with an IC_{50} value of 4.99 μM through compound screening followed by structure-based medicinal chemistry [228, 229]. Modest antiviral activity in cells (EC_{50} = 19.90 μM) was observed. Further modification of ML300 led to a more potent inhibitor CCF0058981 with an IC_{50} of 68 nM as well as an EC_{50} of 497 nM against SARS-CoV-2 [229].

4.2.7. Pyrimidine analogs—High-throughput screen identified pyrimidine compound carmofur to be an inhibitor of SARS-CoV-2 M^{pro} (IC_{50} = 1.82 μM) [40], which modestly inhibited cellular replication of SARS-CoV-2 [230]. The structure of SARS-CoV-2 M^{pro} in complex with carmofur was determined [230]. Subsequent studies revealed that carmofur is a nonspecific inhibitor, and it also showed sub-μM activities against SARS-CoV-2 PL^{pro} and other $3CL^{pro}$ [65, 217]. Another pyrimidine-containing compound **23** was discovered as a potent SARS-CoV-2 M^{pro} inhibitor with an IC_{50} of 20 nM, using virtual screening [231, 232]. Compound **23** was able to inhibit cellular SARS-CoV-2 proliferation with an EC_{50} of 0.84 μM. Its derivative compound **19** showed significantly increased antiviral activity against SARS-CoV-2 (EC_{50} = 80 nM) [233]. Several other pyrimidine and related compounds were also identified to be M^{pro} inhibitors with low-μM IC_{50}s [166, 207, 234–237].

4.2.8. Acrylamide and related compounds—Through screening DNA-encoded compound libraries, compound **1e** with an acrylamide group (Figure 13) was identified as a novel covalent inhibitor of SARS-CoV and -CoV-2 M^{pro} (IC_{50} = 3.5 and 2.0 μM) [238]. However, it showed only weak anti-SARS-CoV-2 activity in cells with an EC_{50} of 33 μM. In addition, LY1 was found to be a dual inhibitor of SARS-CoV-2 M^{pro} (IC_{50} = 0.12 μM) and PL^{pro} (IC_{50} = 0.99 μM) [239]. It can inhibit the viral proliferation with an EC_{50} of 3.9 μM. Several other non-peptidic acrylamide- [240, 241], chloroacetamide- [242] and vinyl sulfonamide-based [243] covalent inhibitors of SARS-CoV and SARS-CoV-2 M^{pro} were also reported with low or untested antiviral activities.

4.2.9. Isatin analogs—Isatin compounds were previously found to be potent inhibitors of related rhinovirus $3CL^{pro}$ [244], with its keto group forming a covalent bond with the active site cysteine residue. Isatin compounds were evaluated for their ability to inhibit M^{pro} [245–248]. Compounds **4o** and **5f** (Figure 13) showed strong inhibition of SARS-CoV M^{pro} with IC_{50}s of 0.95 and 0.37 μM, respectively [245, 246]. Recently, isatin compound **5f** was found to inhibit SARS-CoV-2 M^{pro} (IC_{50} = 45 nM) [248]. No cellular antiviral activities of these compounds were reported. Given the similarities between rhinovirus $3CL^{pro}$ and M^{pro}, these isatin inhibitors of M^{pro} might covalently bind to Cys145.

4.2.10. Metal-containing inhibitors—Transition metal complexes were reported to potently inhibit SARS-CoV and SARS-CoV-2 M^{pro}, including 1-hydroxypyridine-2-thione zinc, bis(*L*-aspartato-N,O) zinc(II) ethanate (JMF1586), thimerosal and phenylmercuric acetate, auranofin, and Re(I) tricarbonyl complex (Figure 14) [249–255]. X-ray crystallographic studies showed that His41 and C145, the catalytic dyad of M^{pro}, chelate Zn^{2+} of JMF1586 [250]. Mechanism of inhibition of other transition metal complexes could similarly involve the formation of a coordination bond(s) with Cys145 and/or His41. No cellular antiviral activities of these compounds were reported.

4.2.11. Triazine compounds—Trisubstituted triazine compound S-217622 (Figure 15), a non-covalent, potent inhibitor of SARS-CoV-2 M^{pro} (IC_{50} = 13 nM), was discovered from virtual screening followed by medicinal chemistry optimization [256]. It selectively inhibited SARS-CoV-2 M^{pro} over the human proteases. S-217622 showed potent antiviral activities in Vero cells against various strains of SARS-CoV-2 with EC_{50} values of 0.29–0.50 μM. Oral administration of S-217622 significantly reduced the titers of SARS-CoV-2 in mice. It also possesses a good pharmacokinetic profile with $T_{1/2}$ of 2.4 h and a high oral bioavailability of 96.7% in rats, as well as a good safety profile in clinical trials [257]. S-217622, renamed to be ensitrelvir, has been approved in Japan to treat COVID-19.

4.2.12. Other miscellaneous compounds—Other miscellaneous compounds were identified as M^{pro} inhibitors. Trisubstituted piperazine compound GC-14 was found to be a SARS-CoV-2 M^{pro} inhibitor with an IC_{50} of 0.40 μM as well as a high selectivity over human cysteine proteases [258]. It suppressed replication of SARS-CoV-2 in Vero cells with an EC_{50} of 1.1 μM without cytotoxicity. Replacement of the nicotinoyl group in GC-14 with a chloroacetyl group yielded the covalent inhibitor GD-9 with a 2-fold increase in enzyme inhibition, but it showed a ~2-fold decreased antiviral activity together with significant cytotoxicity [259]. 1,4-Naphthoquinone compound **15** was found to potently inhibit SARS-CoV-2 M^{pro} (IC_{50} = 72 nM) but moderately blocked the viral replication in Vero cells (EC_{50} = 4.55 μM) [260].

Screening of a DNA-encoded library yielded compound CDD-1976 as a potent SARS-CoV-2 M^{pro} inhibitor with a K_i of 37 nM, which inhibited the virus replication with an EC_{50} of 2.50 μM [261]. Compound ALG-097111 was reported to be a potent SARS-CoV-2 M^{pro} inhibitor with an IC_{50} of 7 nM as well as a high selectivity over cathepsin L [262]. It showed significant cellular (EC_{50} = 200 nM) and in vivo antiviral activity against SARS-CoV-2. However, the structure of ALG-097111 has not been disclosed. From virtual screening, compounds Z1244904919 and Z1759961356 were found to be inhibitors of SARS-CoV-2 M^{pro} (IC_{50} = 0.73 and 0.69 μM), which suppressed the viral replication in Vero cells with EC_{50}s of 4.98 and 8.52 μM, respectively [263]. Walrycin B, a strong inhibition against SARS-CoV-2 M^{pro} (IC_{50} = 0.26 μM), demonstrated an EC_{50} of 3.55 μM against SARS-CoV-2 replication in Vero cells, but it showed a high cytotoxicity (CC_{50} = 4.25 μM) [173].

Several FDA-approved drugs were found to be SARS-CoV and/or -CoV-2 M^{pro} inhibitors. Tyrosine-kinase inhibitor masitinib (Figure 16), an anticancer drug, inhibited SARS-CoV-2 M^{pro} (IC_{50} = 2.5 μM) and the viral replication (EC_{50} = 3.2 μM) [264]. Oral administration of masitinib significantly reduced the viral loads in the lungs and noses and prolonged

the survivals of SARS-CoV-2 infected mice. Phosphodiesterase inhibitor dipyridamole (an antiplatelet drug) [186], BCL-2 inhibitor venetoclax (an anticancer drug) [176], and cinacalcet (a hypercalcemia drug targeting calcium sensing receptor) [176] were also reported to inhibit SARS-CoV-2 M^{pro} with IC_{50}s of 0.60, 3.18, and 5.99 μM, while they showed more potent cellular antiviral activities (EC_{50} = 0.1, 1.18, and 2.93 μM). Interestingly, dipyridamole was used in the clinic to treat severely ill COVID-19 patients [265]. Manidipine (an antihypertension drug) was reported to be an inhibitor of SARS-CoV-2 M^{pro} with an IC_{50} of ~5 μM [93]. But other researchers found that manidipine had only weak or no inhibitory activity [65, 266].

Some other compounds were reported to inhibit SARS-CoV and/or -CoV-2 M^{pro}, but their antiviral activities were weak or unreported [40, 47, 65, 106, 134, 138, 141, 160, 163, 166, 173, 183, 197, 206, 209, 217, 218, 226, 249, 251, 266–318].

5. M^{pro}-inhibitor interactions

More than 100 crystal structures of M^{pro} in complex with its inhibitors have been determined with most of them binding to the active site. Upon complexation with these active-site inhibitors, the overall structure of M^{pro} is little changed. M^{pro} interactions with three representative covalent peptidomimetic inhibitor are shown in Figure 17, which could facilitate future rational inhibitor design.

Compound **11a** (Figure 3), an aldehyde-based inhibitor, binds to the substrate-binding pocket of SARS-CoV-2 M^{pro}, with the aldehyde group forming a covalent bond with Cys145 (Figure 17a, b) [41]. The binding is further stabilized by a network of hydrogen bonds between the newly formed hydroxyl group and the “oxyanion hole” residues Cys145, Gly143, Thr26 and Asn142. There are hydrophobic interactions and a hydrogen bond interaction between the backbone of **11a** and His164. The γ-lactam P1 moiety mimics the substrate glutamine side chain and is located in the S1 pocket of M^{pro} with favorable hydrophobic interactions and hydrogen bonds with Phe140 and Glu166. The cyclohexyl group of the inhibitor fits snugly into the S2 pocket surrounded by Met49, Tyr54, Met165, Asp187, Arg188, and His41. The indole fragment has favorable hydrophobic as well as hydrogen bond interactions with Pro168, Gln189 and Glu166.

Ketone-based peptidomimitic inhibitor **13b** (Figure 4) forms a covalent bond with Cys145 to produce a thiohemiketal (Figure 17c, d), with its hydroxyl group stabilized by a hydrogen bond with His41 [32]. The benzyl amide moiety occupies the S1’ pocket with favorable hydrophobic and hydrogen bond interactions with Gly143, Ser144 and Cys145. The P1 γ-lactam is similarly in the S1 pocket of M^{pro} with favorable hydrophobic and hydrogen bonds with Phe140, Glu166 and His163. The cyclopropylmethyl side chain of the inhibitor occupies the S2 pocket, while the pyridone moiety resembles the binding of the substrate backbone, forming hydrogen bonds with Glu166. Although the terminal *tert*-butyloxycarbonyl (Boc) group does not fit nicely in the S4 pocket, its interactions with the protein contribute to the binding of compound **13b**, as removal of the Boc group significantly reduced the inhibitory activity [32].

Nirmatreivir/PF-07321332 is a nitrile-based inhibitor of M^{pro} (Figure 6), with its nitrile group covalently binding to Cys145 and forming a thioimidate adduct (Figure 17e, f) [86]. Similarly, its γ-lactam moiety fits well in the S1 pocket with favorable hydrophobic and hydrogen bond interactions with His163, Phe140 and Glu166. The 6,6-dimethyl-3-azabicyclo[3.1.0]hexane moiety is located in the S2 pocket of M^{pro}, with mostly hydrophobic interactions with Met49, His41, and Asp187. The tri-fluoroacetamide group, along with the *tert*-butyl side chain, occupies the S4 pocket with favorable hydrophobic interactions with Gln189, Met165, and Pro168.

Protein-inhibitor interactions of four representative non-peptidic inhibitors of M^{pro} are shown in Figure 18. Compound 23R (Figure 7) is a potent, non-covalent inhibitor of M^{pro} (Figure 18a, b) [143]. Its furan-carboxyl moiety is located in the S1' pocket of M^{pro} with both hydrophobic and hydrogen bond interactions. The 3-pyridine ring occupies the S1 pocket with favorable hydrophobic interacts as well as hydrogen bonds with His163 and Ser144. The biphenyl moiety fits nicely into the S2 pocket with hydrophobic and π-π stacking interactions. There is another hydrogen bond between the other amide oxygen of the inhibitor and Glu166. The chiral α-methylbenzyl group is extended into the S4 pocket of M^{pro} with mostly hydrophobic interactions.

S-217622 (Figure 15) is a potent, non-covalent inhibitor of M^{pro} [256]. The 1-methyl-1*H*-1,2,4-triazole group of S-217622 occupies the S1 pocket with favorable hydrophobic and hydrogen bond interactions with Ser144, His163 and Asn142 (Figure 18c, d). Its central triazine-2,4-dione core resides on a shallow platform linking the S1 and S2 pockets, forming a network of direct or water-mediated hydrogen bonds with Asn142, Ser144, Cys145, and Glu166. There might be also favorable hydrophobic or other interactions between the aromatic ring and the -SH of Cys145 (with the distance of ~5 Å). The 2,4,5-trifluorobenzylic substituent fits well in the S2 pocket with favorable hydrophobic and π-π stacking (with His41) interactions. The indazole moiety of S-217622 is situated in the S1' pocket with stabilizing hydrophobic as well as hydrogen bond interactions with Thr26. The -NH- linker group interacts with His41 and Gln189 through water-mediated hydrogen bonds. Furthermore, this fragment may establish hydrophobic interactions with Met49 and Thr25.

Masitinib (Figure 16) is a moderate, non-covalent inhibitor SARS-CoV-2 M^{pro}, with its long, linear body binding across the S1 and S2 pockets of the protein (Figure 18e, f) [264]. Its thiazole-pyridine moiety is located the S1 pocket with hydrophobic interactions as well as a hydrogen bond with His163. The -NH- linker group forms a hydrogen bond with His164. The toluene fragment is situated in the S2 pocket of M^{pro} with hydrophobic and π-π stacking (with His41) interactions. Its benzamide and terminal *N*-methylpiperaze moieties protrude from the S2 pocket further into the protein, with mostly hydrophobic interactions as well as hydrogen bonds with His41 and Thr24.

Natural flavonoid compound myricetin (Figure 8) was found to be oxidized by O_2 to become an *ortho*-quinone, which is a covalent inhibitor of SARS-CoV-2 M^{pro} with a unique binding mode (Figure 18g, h) [152]. The *ortho*-quinone acts as an electrophile and forms a covalent bond with Cys145. The molecule is mainly located in the S1' and S2 pockets.

The hydroxyl (or its oxidized form) groups in the pyrogallol fragment form multiple hydrogen bond interactions with Thr26 and Cys145. The pyrogallol ring might also have hydrophobic interactions with Leu27, Asn28, Asn142, Gly143 and Ser144. In addition, the hydroxyl-substituted chromone moiety has hydrogen bond interactions with Gln189 and His164. There are also favorable π–π stacking and hydrophobic interactions between the chromone core and His41, Met165, Asp187 and Arg188.

6. M^{pro} inhibitors that are FDA-approved or in clinical trials

Nirmatrelvir in combination with ritonavir (brand name Paxlovid) has been approved by FDA for the treatment of mild-to-moderate SARS-CoV-2 infected patients. This clinical trial (NCT04960202) included 2246 adult patients (1120 treated and 1126 controls) with confirmed SARS-CoV-2 infection within 5 days. Treatment with Paxlovid lowered the risk of progression to severe diseases by 89% without obvious safety issues [319]. A large retrospective study including 180,351 high-risk COVID-19 patients revealed that treatment with Paxlovid showed high efficacies in reducing hospitalization and mortality [320]. Another anti-SARS-CoV-2 drug, ensitrelvir fumaric acid (brand name Xocova), has been granted emergency regulatory approval in Japan. As shown in Table 1, a handful of other M^{pro} inhibitors (including several with their structures undisclosed) have been in different stages of clinical trials (data from clinicaltrials.gov).

7. Conclusion and perspectives

The Covid-19 pandemic caused by SARS-CoV-2 has been an unprecedented catastrophe of the global health in modern history with millions of mortalities and morbidities. It has also resulted in enormous economic losses worldwide. Thanks to expedited development and deployment of effective vaccines and antiviral drugs against SARS-CoV-2, the pandemic has been largely over within 3 years. However, with continuous viral evolution as well as possible emergence of a new coronavirus, drug discovery targeting SARS-CoV-2 and related viruses is needed.

M^{pro} is a validated drug target for the coronavirus family because of its essential role in the life cycle of coronaviruses. M^{pro} is a highly conserved protein during evolution [321], which renders a high likelihood of developing a broadly active anti-coronavirus drug or expediting drug discovery against other coronaviruses. Different from the vaccines targeting the spike protein (with frequent mutations), Paxlovid has retained effectiveness against the original to the recent Omicron strains of SARS-CoV-2 [321]. However, treatment with an M^{pro} inhibitor may pose a selective pressure to generate drug resistance, as recently observed nirmatrelvir-resistant strains of SARS-CoV-2 [322]. Therefore, continued research and development on M^{pro} inhibition are warranted. Indeed, the rapid development of nirmatrelvir within 2 years has been based on a lead compound against M^{pro} of SARS-CoV identified in 2003 [323].

This article represents a comprehensive review of small molecule inhibitors of SARS-CoV and -CoV-2 M^{pro} since 2003. Currently, the highly potent inhibitors are mostly peptidic/peptidomimetic compounds with an electrophilic “warhead” to covalently bind Cys145.

Aldehyde and chloroacetamide group exhibit a high chemical reactivity, but they are often associated with non-specific binding, off-target effects and cytotoxicity. With a reduced and tunable reactivity, ketone, epoxide and Michael acceptor groups represent a balanced choice for the warhead and have been successfully utilized in numerous drugs, such as telaprevir, carfilzomib and neratinib. Due to its weak reactivity, nitrile has been rarely used for this application, but it generally possesses improved chemical and metabolic stabilities as well as target specificity, which are critical for clinical use. As for the main body of the inhibitor, M^{pro}'s substrate sequence as well as its X-ray structures can be used to guide peptidic/peptidomimetic inhibitor design and facilitate optimization of the activity and selectivity. Non-peptidic inhibitors were mostly from compound screening, with fewer compounds having potent biochemical and antiviral activities. Since non-peptidic compounds tend to resist enzyme-mediated hydrolysis with improved pharmacokinetics, more work on developing non-peptidomimetic M^{pro} inhibitors is desirable.

In addition, other technologies could be explored to counteract M^{pro}. Proteolysis-targeting chimera (PROTAC) technology [324] has been developed for target protein degradation, which complements and is often similar to protein inhibition. Several PROTAC molecules with antiviral activities have been reported [325, 326]. PROTAC has other potential benefits, such as sub-stoichiometric activity, more selectivity and retained activity against a mutant target protein. But unlike a typical small molecule inhibitor, PROTAC molecules are generally less drug-like with a large molecular mass and various issues in pharmacokinetics and pharmacodynamics. Artificial intelligence (AI) has been rapidly evolving in recent years and could become a powerful tool for drug discovery. Given the large amount of data of in vitro, in vivo and clinical inhibition of cysteine proteases, AI could significantly contribute to the antiviral drug discovery against SARS family of coronaviruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SARS-CoV	severe acute respiratory syndrome-associated coronavirus
M^{pro}	main protease
RdRp	RNA-dependent RNA polymerase
PL^{pro}	papain-like protease
ACE2	angiotensin-converting enzyme 2
ORF	open reading frame
nsps	non-structural proteins

ERGIC	endoplasmic reticulum-Golgi intermediate compartment
CPE	cytopathic effect
HCV	hepatitis C virus
EGFR	epidermal growth factor receptor
Boc	tert-butyloxycarbonyl
EUA	Emergency Use Authorization
PROTAC	proteolysis-targeting chimera
HTS	high-throughput screening
AI	artificial intelligence

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Highlights

- Main protease (M^{pro}) is essential for SARS-CoV and -CoV-2 and therefore an antiviral drug target.
- Function, structure and mechanism of catalysis of M^{pro} are reviewed.
- Small-molecule inhibitors of M^{pro} and their biological activities are summarized.
- Protein-inhibitor interactions of representative compounds are described.

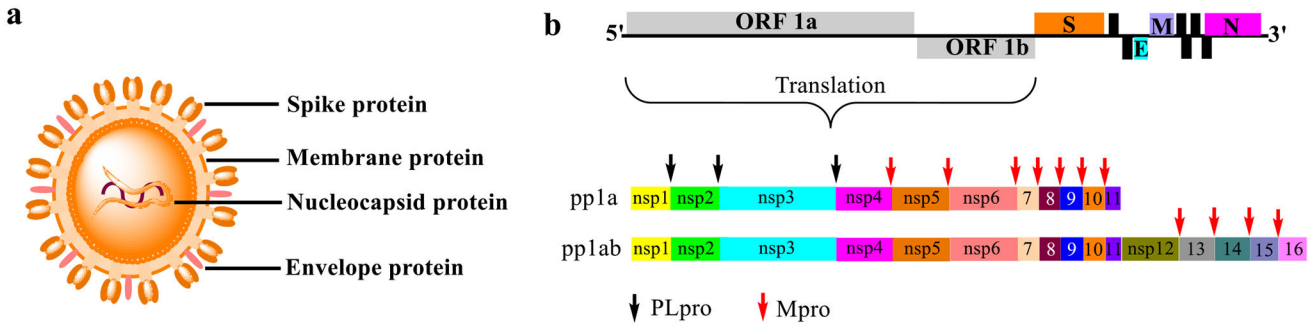
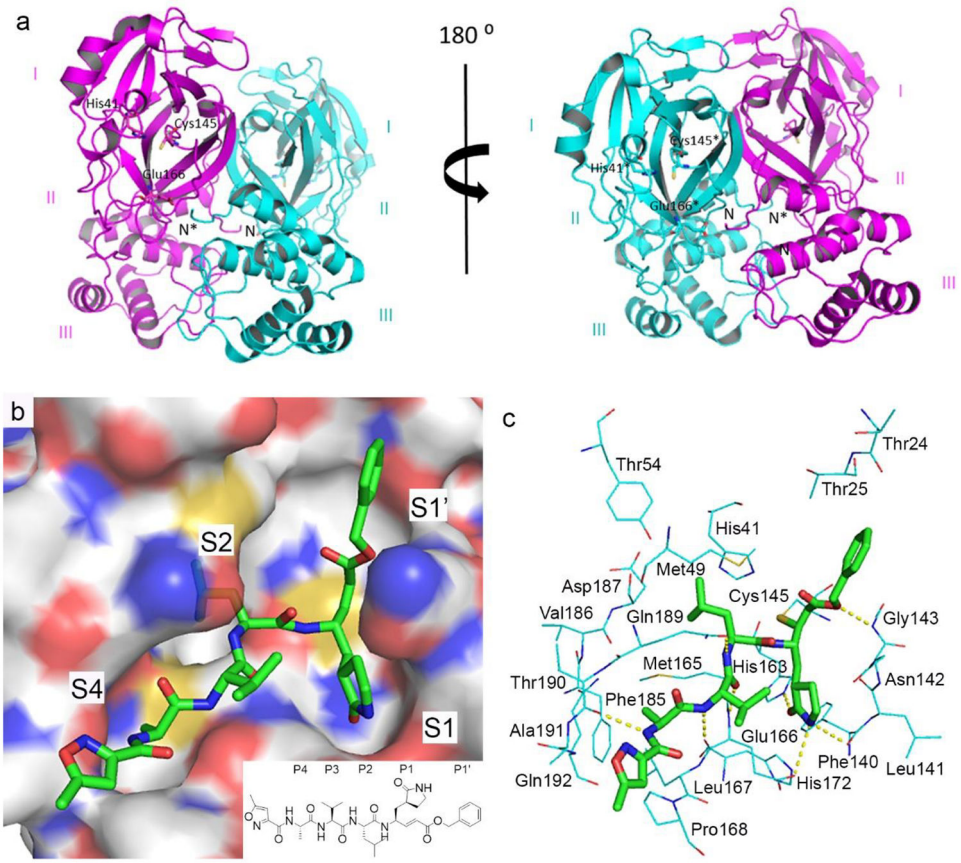


Figure 1.

Schematic illustrations of (a) SARS coronavirus; and (b) The RNA genome of SARS coronavirus containing a 5'-untranslated region, 3'-untranslated region and open reading frames (ORF). ORF1a/b encode non-structure proteins (nsp) and other sequences encode the spike protein (S), envelope protein (E), membrane protein (M), nucleocapsid protein (N) and several accessory proteins (in black). The viral polyproteins (pp1a and pp1ab) translated from ORF1a/b are site-specifically cleaved by the viral M^{pro} (nsp5, red arrows) and PL^{pro} (nsp3, black arrows) to give viral non-structure proteins.



d

Substrates	Sequences (P6…P1/P1'…P6')
nsp4/5	TSAVLQ/SGFRKM
nsp5/6	SGVTFQ/SAVKRT
nsp6/7	KVATVQ/SKMSDV
nsp7/8	NRATLQ/AIASEF
nsp8/9	SAVKLQ/NNELSP
nsp9/10	ATVRLQ/AGNATE
nsp10/12	REPMLQ/SADAQS
nsp12/13	PHTVLQ/AVGACV
nsp13/14	NVATLQ/AENVTG
nsp14/15	TFTRLQ/SLENVA
nsp15/16	FYPKLQ/SSQAWQ

e

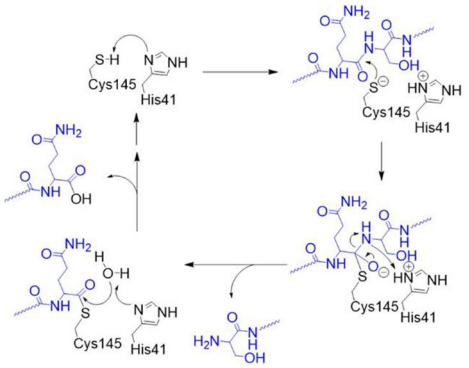


Figure 2.

SARS-CoV-2 M^{pro} structure, substrates and mechanism of catalysis. (a) The homodimeric structure of SARS-CoV-2 M^{pro} (PDB: 6Y2G), with one monomer shown in cyan and the other in magentas. (b) The active site of M^{pro}-N3 complex (PDB: 6LU7), with M^{pro} shown as an electrostatic surface and N3 as a tube model with C atoms in green; (c) The M^{pro}-N3 interactions with hydrogen bonds shown as dashed lines. Cys145 forms a covalent bond with the inhibitor; (d) Sequences of the SARS-CoV-2 M^{pro} substrates; (e) Mechanism of catalysis for M^{pro}.

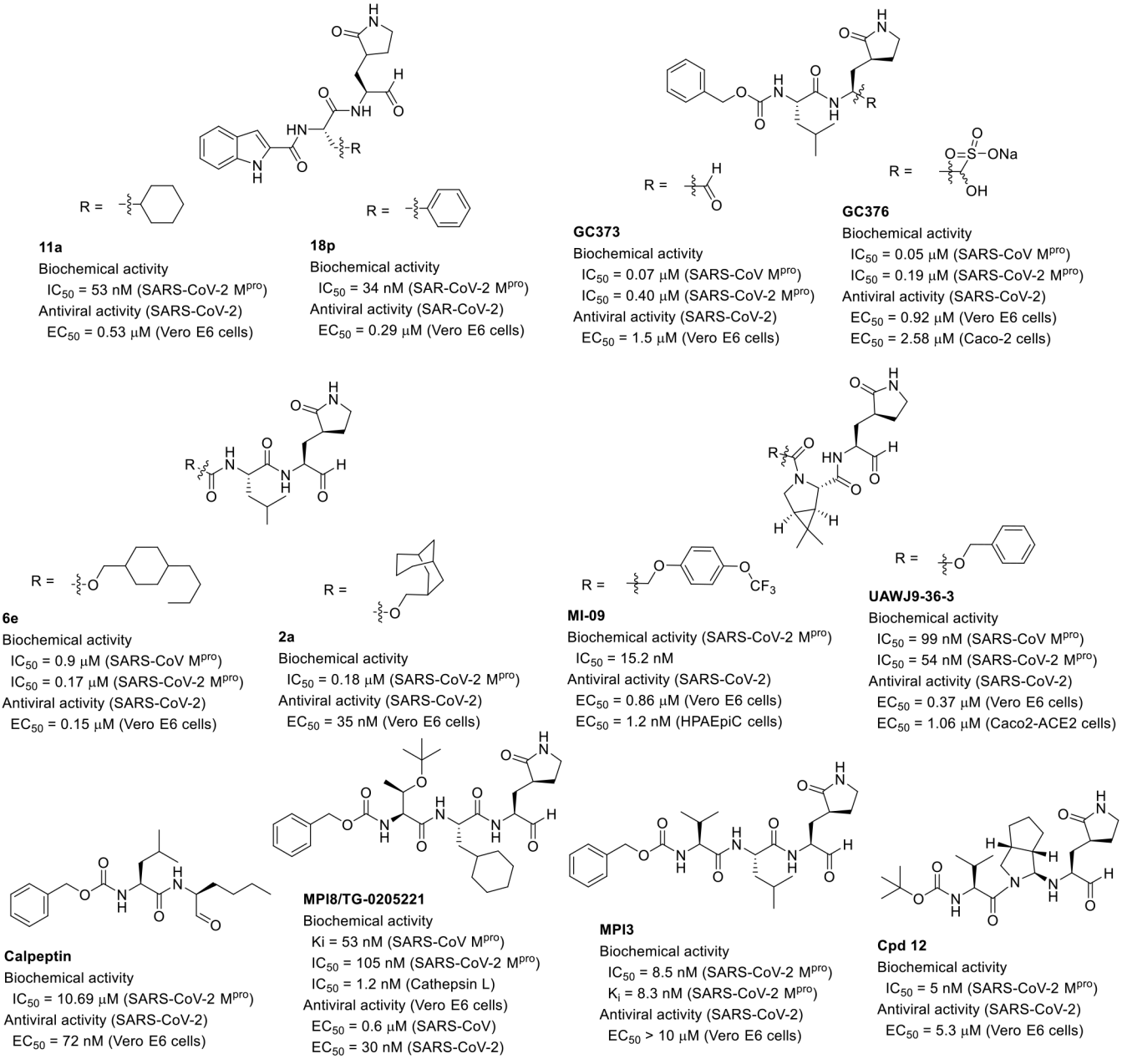


Figure 3.

Structures and biological activities of aldehyde-based peptidomimetic inhibitors of M^{pro}.

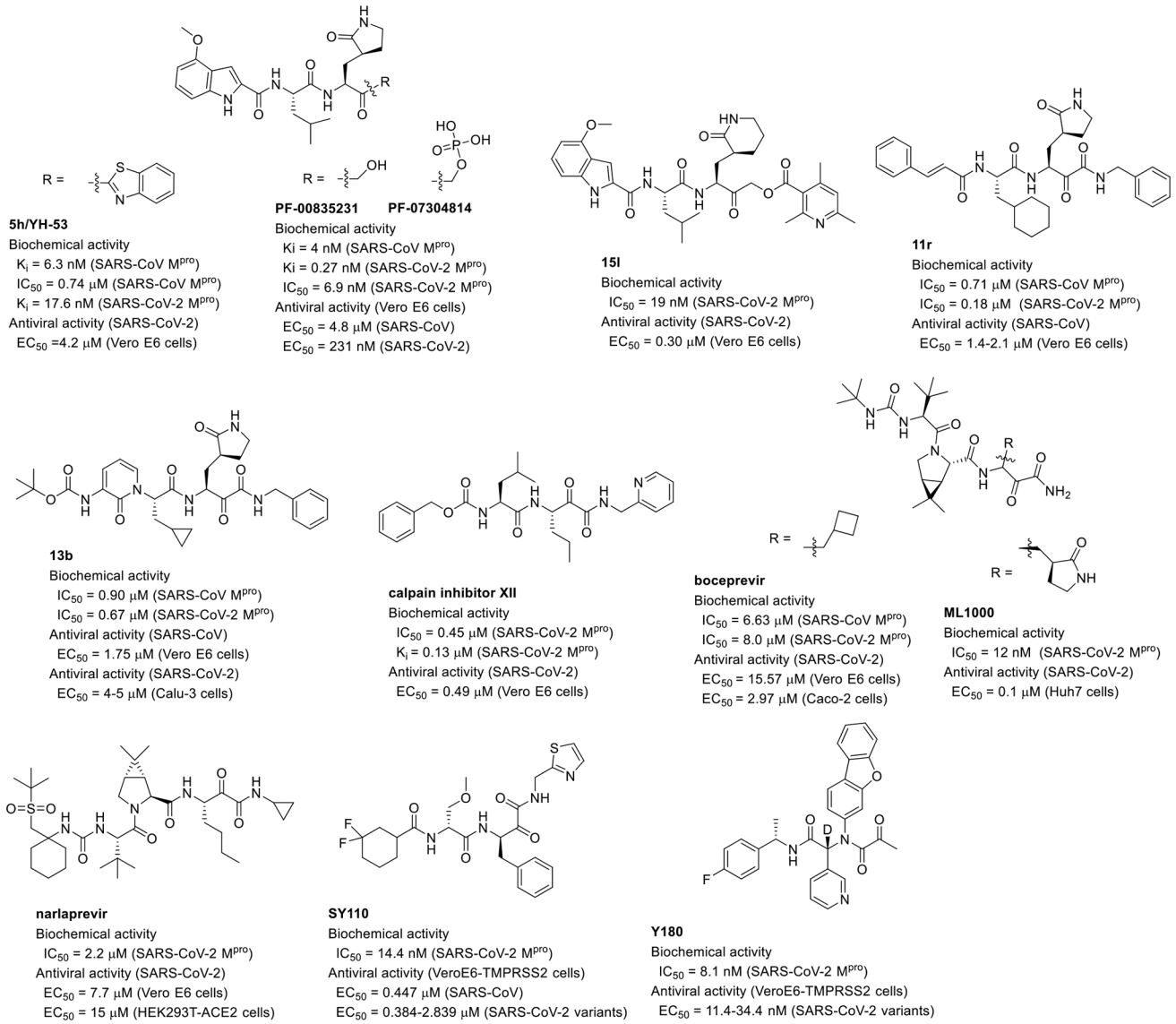


Figure 4.

Structures and biological activities of ketone-based peptidomimetic inhibitors of M^{pro}.

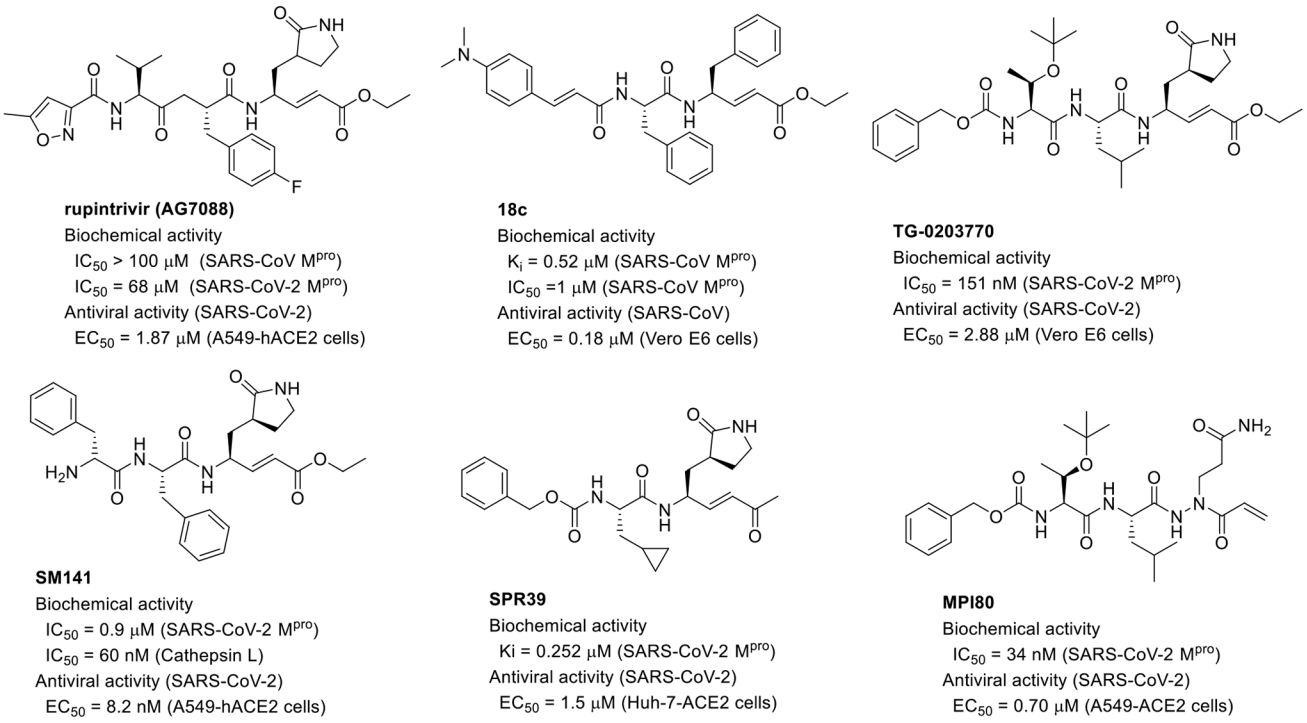


Figure 5.

Structures and biological activities of α, β-unsaturated ester inhibitors of M^{pro}.

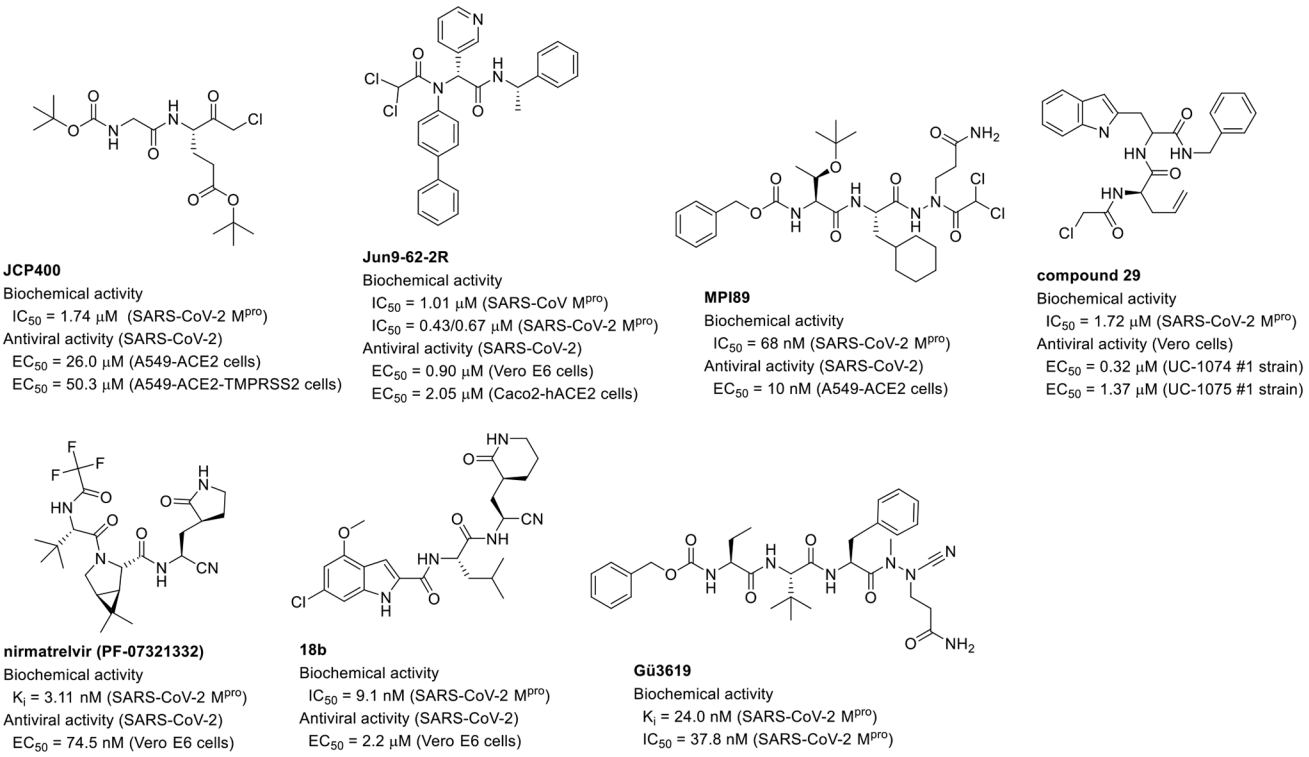
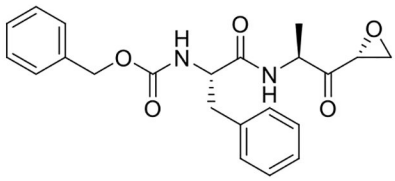


Figure 6.

Structures and biological activities of haloacetyl- and nitrile-based peptidomimetic inhibitors of M^{pro}.



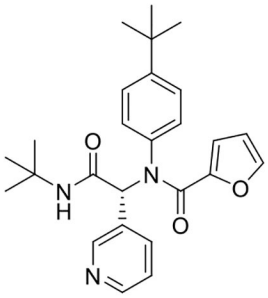
WRR183

Biochemical activity

K_i = 2.2 μM (SARS-CoV M^{pro})

Antiviral activity (SARS-CoV)

EC_{50} = 12 μM (Vero E6 cells)



ML188

Biochemical activity

IC_{50} = 1.5-4.5 μM (SARS-CoV M^{pro})

K_i = 1.6 μM (SARS-CoV M^{pro})

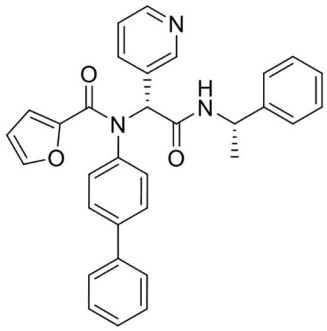
IC_{50} = 2.5 μM (SARS-CoV-2 M^{pro})

Antiviral activity (SARS-CoV)

EC_{50} = 13 μM (Vero E6 cells)

Antiviral activity (SARS-CoV-2)

EC_{50} > 20 μM (racemic, Vero E6 cells)



23R

Biochemical activity

IC_{50} = 0.27 μM (SARS-CoV M^{pro})

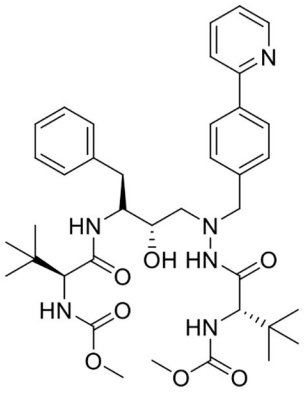
IC_{50} = 0.20 μM (SARS-CoV-2 M^{pro})

K_i = 0.07 μM (SARS-CoV-2 M^{pro})

Antiviral activity (SARS-CoV-2)

EC_{50} = 1.27 μM (Vero E6 cells)

EC_{50} = 3.03 μM (Calu-3 cells)



atazanavir

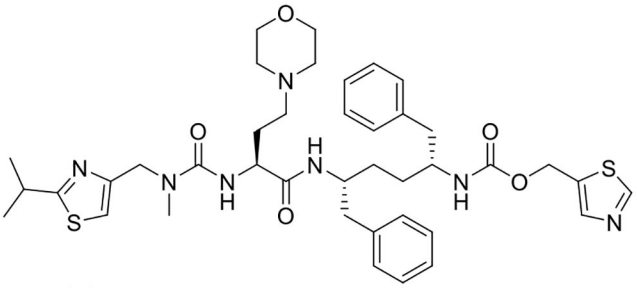
Biochemical activity

K_i = 703 nM (SARS-CoV-2 M^{pro})

Antiviral activity (SARS-CoV-2)

EC_{50} = 2.0 μM (Vero E6 cells)

EC_{50} = 0.32-0.49 μM (Calu3 cells, different variants)



cobicistat

Biochemical activity

IC_{50} = 6.7 μM (SARS-CoV-2 M^{pro})

K_d = 2.2 μM (SARS-CoV-2 M^{pro})

Antiviral activity (SARS-CoV-2)

EC_{50} = 2.74 μM (nanoluciferase reporter assay)

Figure 7.

Structures and biological activities of the other peptidic inhibitors of M^{pro}.

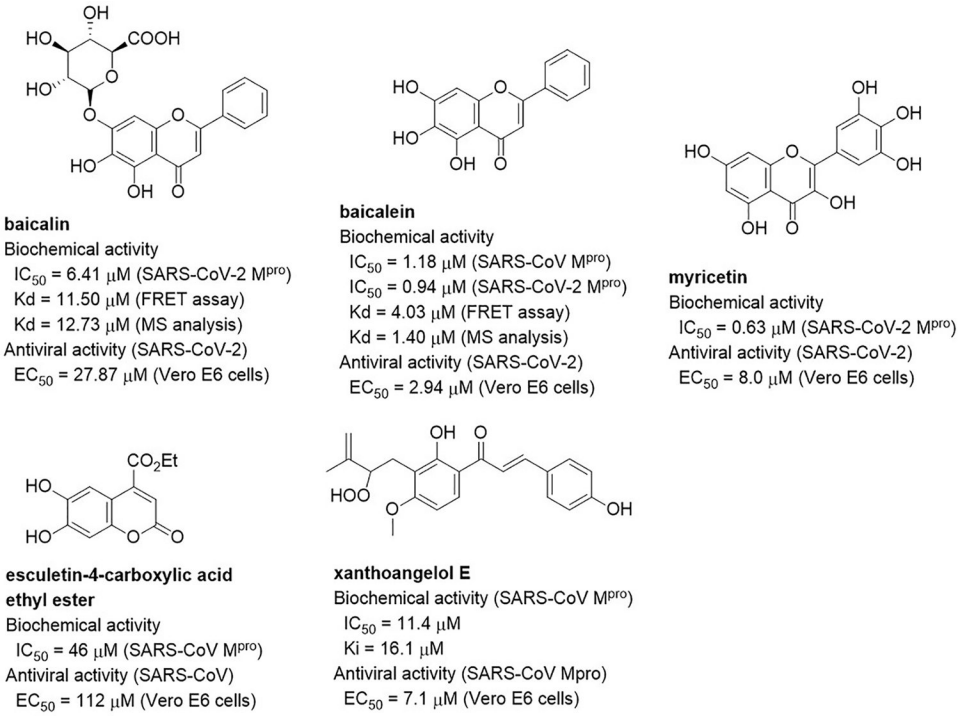
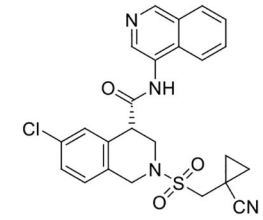
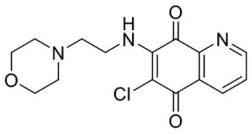


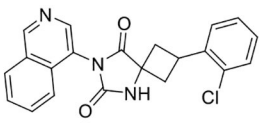
Figure 8.
Structures and biological activities of flavonoid inhibitors of M^{pro}.



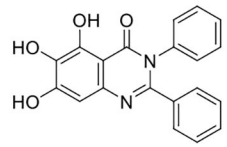
MAT-POS-e194df51-1
Biochemical activity
IC_{50} = 36.8 nM (SARS-CoV-2 M^{pro})
Antiviral activity (SARS-CoV-2)
EC_{50} = 63.8 nM (A549 cells)
EC_{50} = 149 nM (Hela-Ace2 cells)
EC_{50} = 1.15 μM (Calu-3 cells)
Antiviral activity (SARS-CoV-2 variants)
EC_{50} = 0.29-1.52 μM (Hela-Ace2 cells)



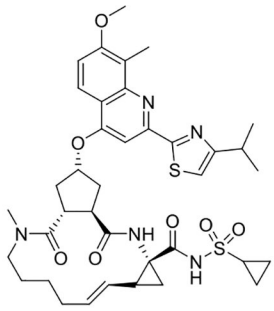
DA-3003-1
Biochemical activity
(SARS-CoV-2 M^{pro})
IC_{50} = 2.63 μM
Antiviral activity (SARS-CoV-2)
EC_{50} = 4.47 μM (Vero E6 cells)



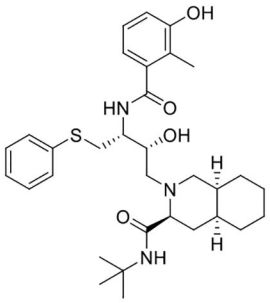
compound 19
Biochemical activity
IC_{50} = 77 nM (SARS-CoV-2 M^{pro})
Antiviral activity (SARS-CoV)
EC_{50} = 390 nM (Vero E6 cells)
Antiviral activity (SARS-CoV-2)
EC_{50} = 77 nM (Vero E6 cells)
EC_{50} = 110 nM (Huh7 cells)



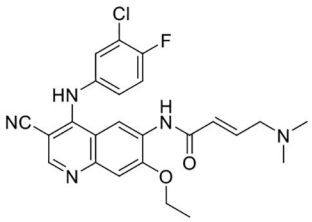
C7
Biochemical activity
IC_{50} = 85 nM (SARS-CoV-2 M^{pro})
Antiviral activity (SARS-CoV-2)
EC_{50} = 1.10 μM (Vero E6 cells)



simeprevir
Biochemical activity
IC_{50} = 2.46-13.74 μM (SARS-CoV-2 M^{pro})
Antiviral activity (SARS-CoV-2)
EC_{50} = 1.40 μM (Vero E6 cells)

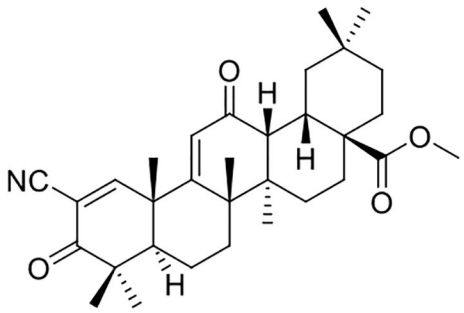


nelfinavir
Antiviral activity (Vero E6 cells)
EC_{50} = 0.048 μM (SARS-CoV)
EC_{50} = 0.77-3.3 μM (SARS-CoV-2)

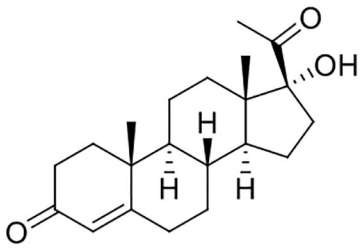


pelitinib
Antiviral activity (SARS-CoV-2)
EC_{50} = 1.25 μM (Vero E6 cells)

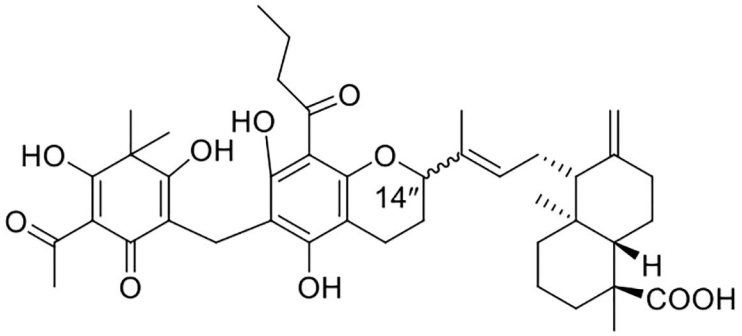
Figure 9.
Structures and biological activities of quinolone and related inhibitors of M^{pro}.



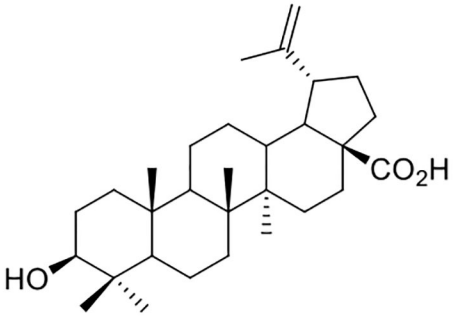
bardoxolone methyl
Biochemical activity
IC_{50} = 5.81 μM (SARS-CoV-2 M^{pro})
Antiviral activity (SARS-CoV-2)
EC_{50} = 0.29 μM (Vero cells)
EC_{50} = 0.20 μM (Calu-3 cells)



hydroxyprogesterone
Biochemical activity
IC_{50} = 2.47 μM (SARS-CoV-2 M^{pro})
Antiviral activity (Vero E6 cells)
EC_{50} = 2.77 μM (SARS-CoV-2)



3 (14″ *S*)
Biochemical activity
Kd = 16.6 μM (SARS-CoV-2 M^{pro})
IC_{50} = 7.5 μM (SARS-CoV-2 M^{pro})
Antiviral activity (SARS-CoV-2)
EC_{50} = 4.5 μM (Vero E6 cells)
EC_{50} = 20.2 μM (Calu-3 cells)



betulinic acid
Biochemical activity
IC_{50} = 10 μM (SARS-CoV M^{pro})
Ki = 8.2 μM (SARS-CoV M^{pro})
Antiviral activity (SARS-CoV)
EC_{50} > 10 μM (Vero E6 cells)

Figure 10.
Structures and biological activities of terpenoid inhibitors of M^{pro}.

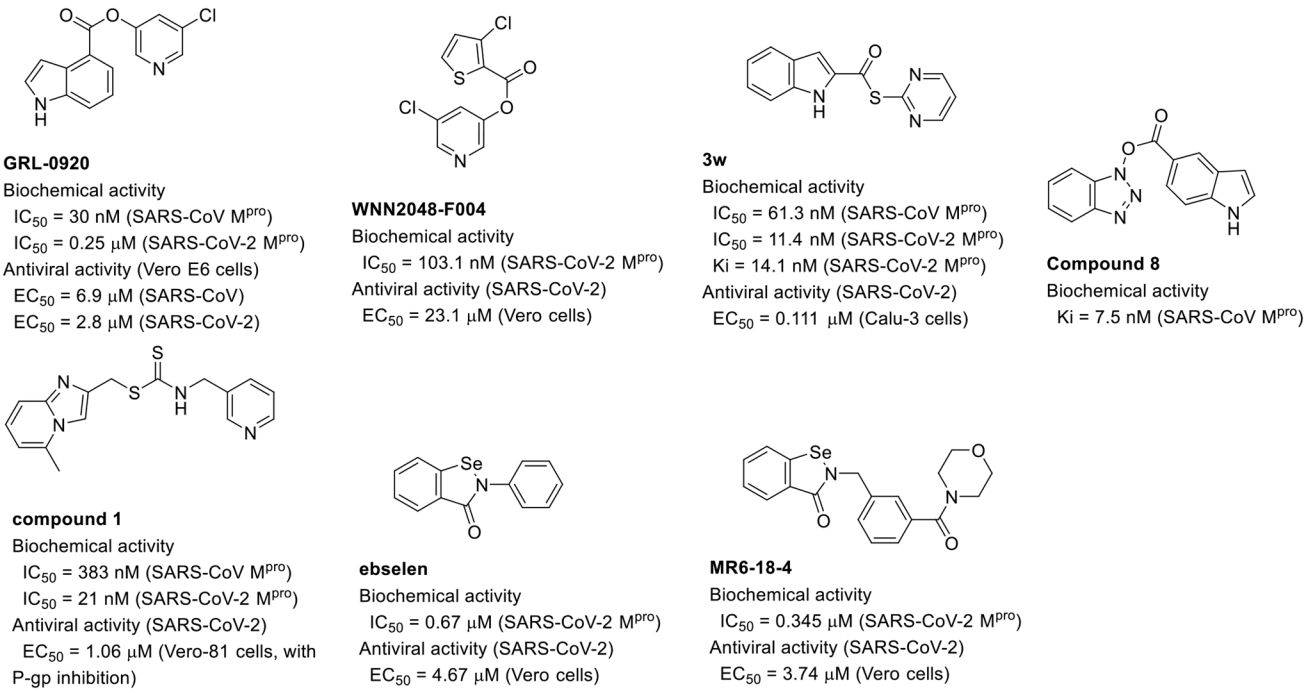


Figure 11.

Structures and biological activities of pyridinyl ester- and ebselen-based inhibitors of M^{pro}.

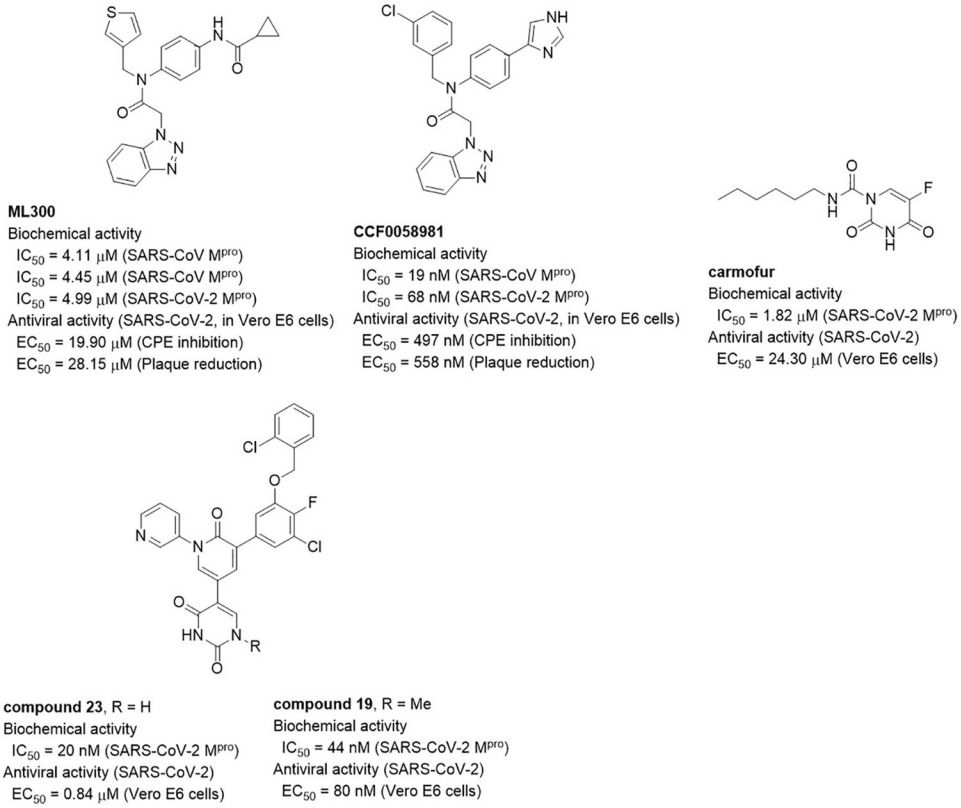


Figure 12.

Structures and biological activities of benzotriazole- and pyrimidine-based inhibitors of M^{pro}.

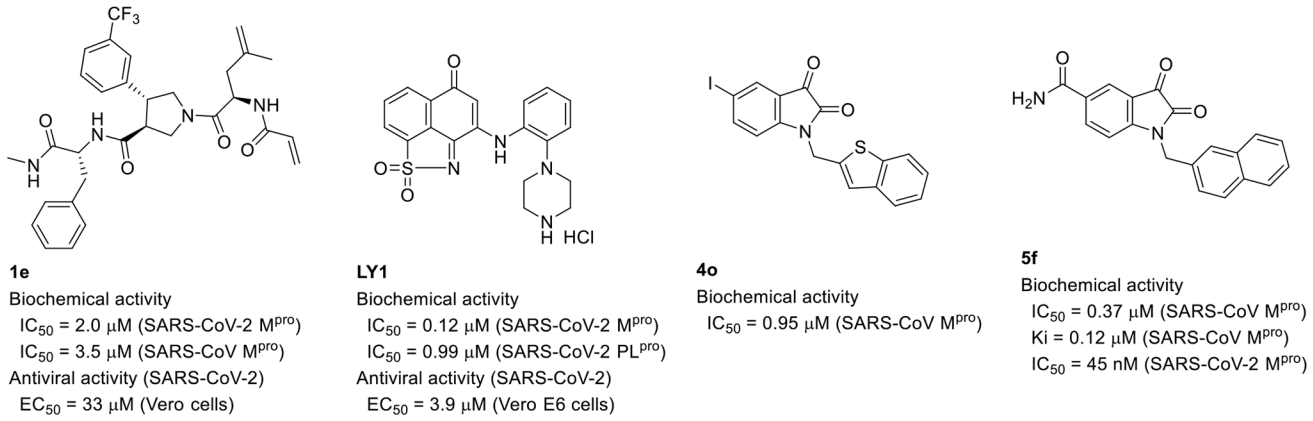


Figure 13.
Structures and biological activities of acrylamide, isatin and related inhibitors of M^{pro}.

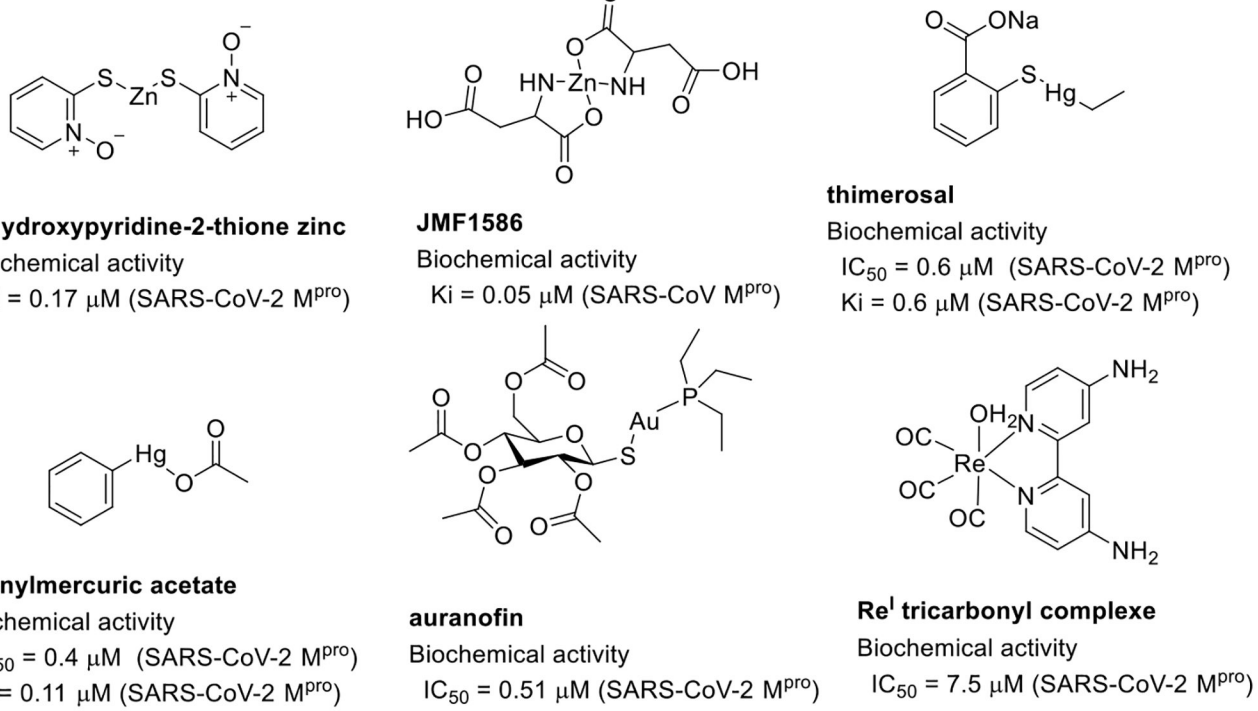


Figure 14.
Structures and biological activities of metal-containing inhibitors of M^{pro}.

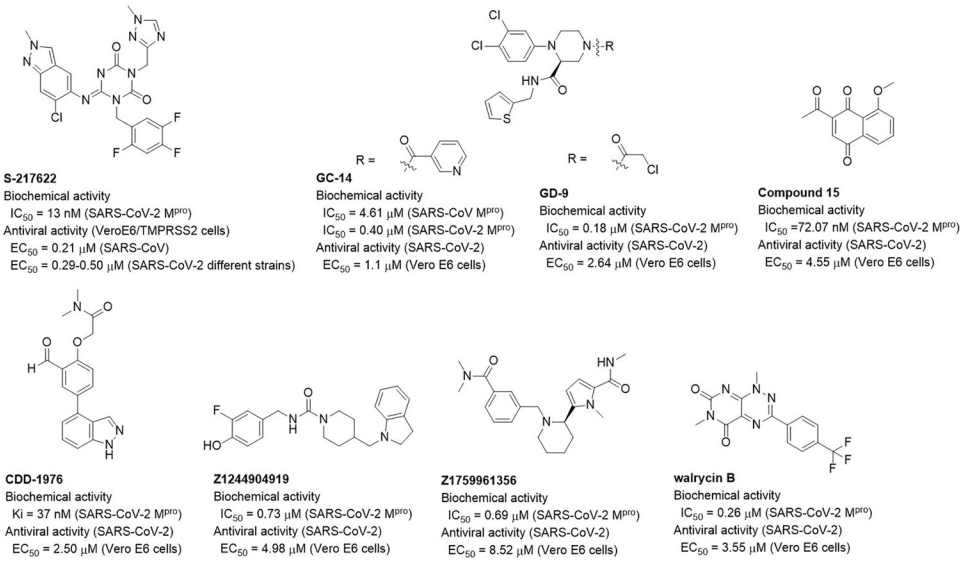


Figure 15.

Structures and biological activities of triazine and other miscellaneous inhibitors of M^{pro}.

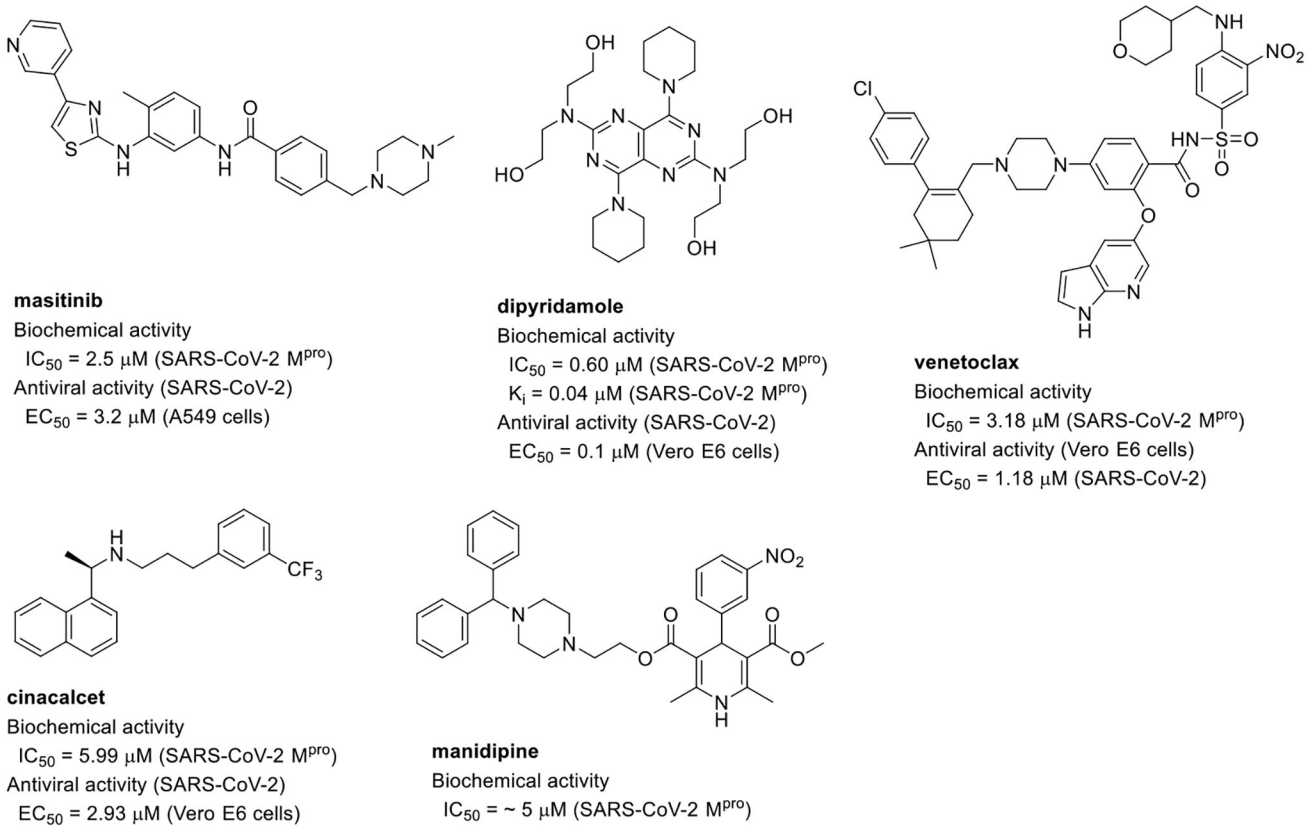


Figure 16.
Structures and biological activities of clinical drugs that inhibit M^{pro}.

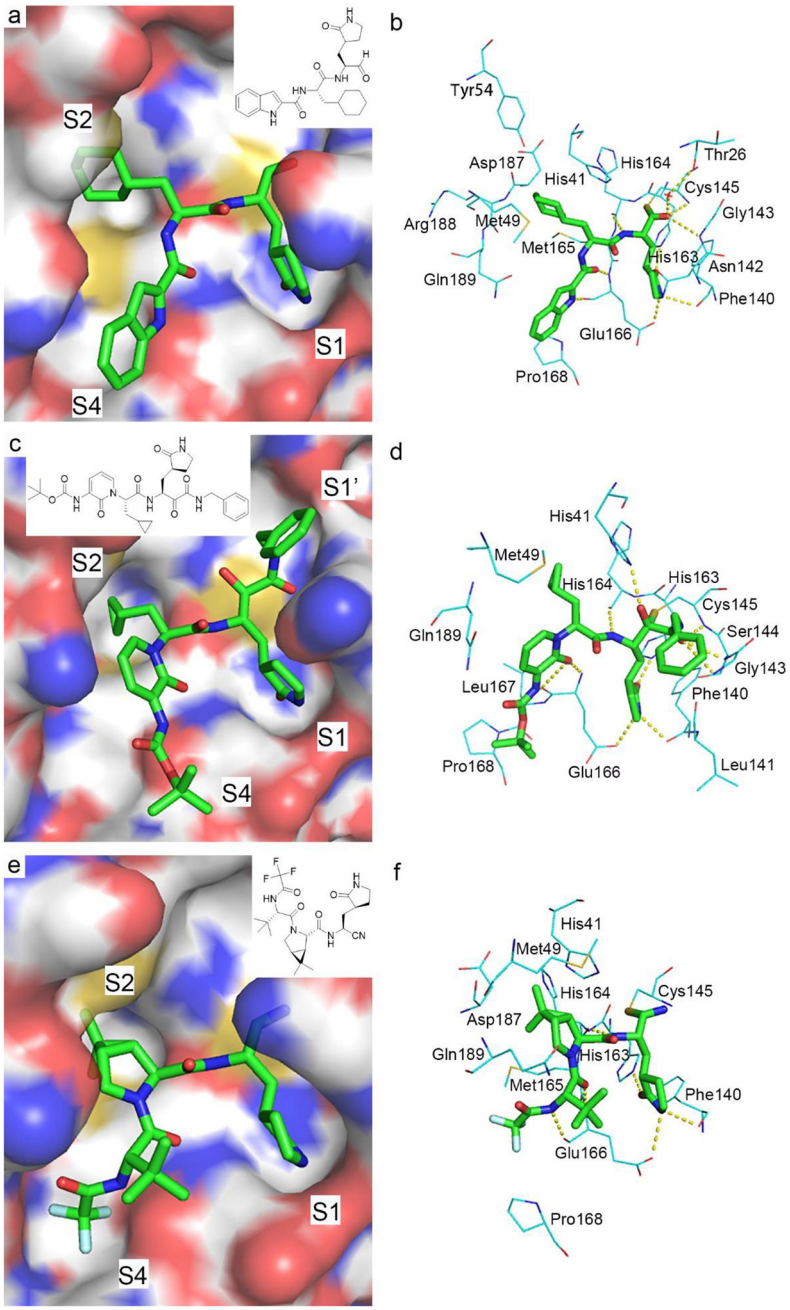


Figure 17.

X-ray structures of M^{pro} in complex with representative peptidomimetic covalent inhibitors. (a, c, e) The active site of M^{pro} (shown as an electrostatic surface) in complex with (a) **11a** (PDB: 6LZE), (c) **13b** (PDB: 6Y2G) and (e) nirmatrelvir (PDB: 7RFW); (b, d, f) The M^{pro}-inhibitor interactions for (b) **11a**, (d) **13b** and (f) nirmatrelvir. Hydrogen bonds are shown as yellow dashed lines.

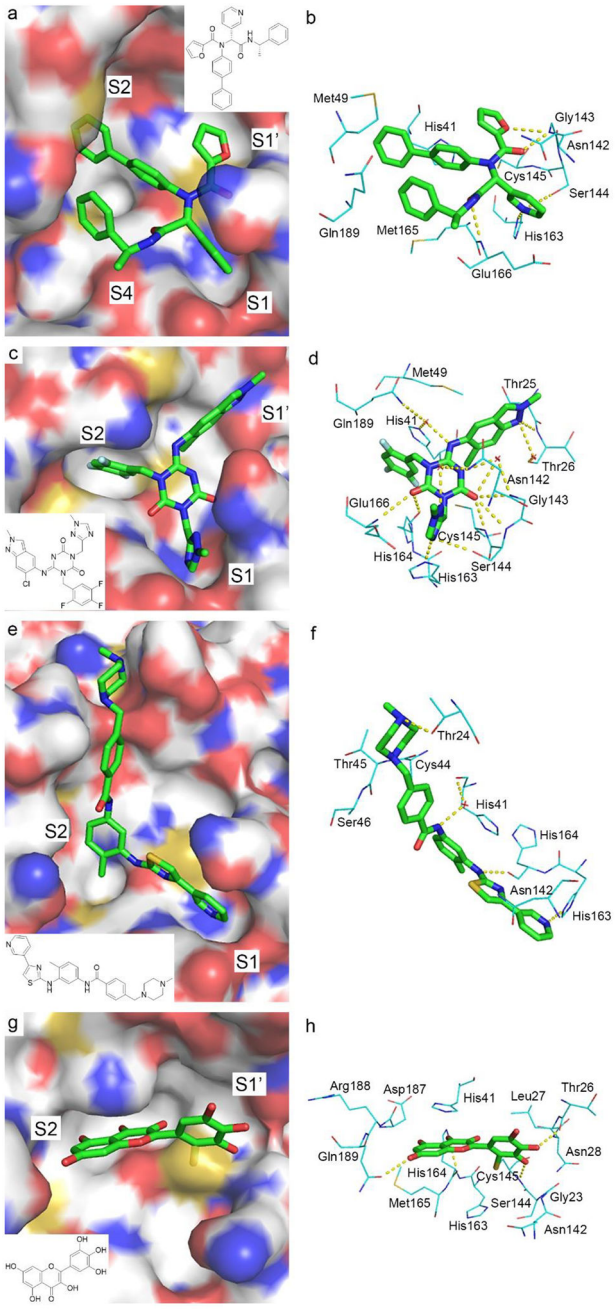


Figure 18.

X-ray structures of M^{pro} in complex with non-peptidic inhibitors. (a, c, e, g) The active site of M^{pro} (shown as an electrostatic surface) in complex with (a) 23R (PDB: 7KX5), (c) S-217622 (PDB: 7VU6), (e) masitinib (PDB: 7JU7) and (g) oxidized myricetin (PDB: 7DPP); (b, d, f, h) The M^{pro}-inhibitor interactions for (b) 23R, (d) S-217622, (f) masitinib and (h) oxidized myricetin. Hydrogen bonds are shown as yellow dashed lines.

Table 1.

SARS-CoV and -CoV-2 M^{pro} inhibitors in clinical trial.

Name	Clinical stage	Clinical trial identifier	Sponsor
ensitrelvir (S-217622) (Figure 15)	Approved in Japan	NCT05897541, NCT05305547, NCT05605093	Shionogi Inc., University of Minnesota
STI-1558	Phase I/III	NCT05523739, NCT05716425	Zhejiang ACEA Pharmaceutical Co. Ltd.
pomotrelvir (PBI-0451)	Phase II	NCT05543707	Pardes Biosciences, Inc.
EDP-235	Phase II	NCT05616728	Enanta Pharmaceuticals, Inc.
ASC11	Phase I	NCT05718518	Ascletis Pharmaceuticals Co., Ltd.
HS-10517	Phase II	NCT05779579	Jiangsu Hansoh Pharmaceutical Co., Ltd.
PF-07304814 (Figure 4)	Phase I	NCT04627532, NCT04535167, NCT05050682	Pfizer
nirmatrelvir (Figure 6) /ritonavir	Approved worldwide	NCT05668091, NCT05576662	Harlan M Krumholz / Stanford University
montelukast	Phase II	NCT04718285	Bahcesehir University
masitinib (AB1010) (Figure 16)	Phase II	NCT05047783	AB Science