Targeting Proteases for Treating COVID-19
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ABSTRACT: The unprecedented pandemic of coronavirus disease 2019 (COVID-19) demands effective treatment for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The infection of SARS-CoV-2 critically depends on diverse viral or host proteases, which mediate viral entry, viral protein maturation, as well as the pathogenesis of the viral infection. Endogenous and exogenous agents targeting for proteases have been proved to be effective toward a variety of viral infections ranging from HIV to influenza virus, suggesting protease inhibitors as a promising antiviral treatment for COVID-19. In this Review, we discuss how host and viral proteases participated in the pathogenesis of COVID-19 as well as the prospects and ongoing clinical trials of protease inhibitors as treatments.

KEYWORDS: SARS-CoV-2, COVID-19, main protease, TMPRSS2, ACE2

1. INTRODUCTION
The ongoing unprecedented pandemic of coronavirus disease 2019 (COVID-19) has resulted in over 5.5 million confirmed cases and over 350 000 deaths reported in over 200 countries, areas, and territories as of May 28, 2020.1 To contain the spread of COVID-19, most governments around the world have taken various measures, such as quarantine, isolation, social distancing, country border shutdown, and so on. Consequently, the COVID-19 pandemic has not only caused the largest global economic recession since the Great Depression but also led to worldwide disruption of education and social activities. According to UNESCO, the school closures, on either a nationwide or local basis in over 190 countries, affected over 90% of the world’s student population in April 2020.2 However, there is currently still no vaccine or specific antiviral medicine to prevent or treat COVID-19.3,4 The urgent need to prevent and treat COVID-19 has bolstered global research on COVID-19 and its causative novel coronavirus.5–8

The highly contagious and pathogenic COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to a broad family of coronaviruses that can infect many birds and mammals, including humans. Similar to the other coronaviruses, SARS-CoV-2 is a positive-sense single-stranded RNA virus, which has a lipid envelope with club-shaped spike (S) proteins protruding from the virion surface.9,10 The infection and the replication cycle of SARS-CoV-2 begin with the binding of its S protein to the angiotensin-converting enzyme 2 (ACE2) receptor on a human cell surface, followed by a structural change of the S protein that enables the fusion of the viral membrane and the cell membrane.3,11 Then, the viral genes can enter the host cell to be replicated, producing more viruses for further viral shedding.12 (Figure 1).

The infection of humans by SARS-CoV-2, as well as the replication cycle of this virus in human cells, depends critically on various proteases, as schematically illustrated in Figure 1. For example, the binding and subsequent cell entry of SARS-CoV-2 are controlled by a wide range of the host proteases.13,14 The viral replication and maturation inside the host cells significantly depends on the viral proteases, such as the main protease (Mpro) and the Papain-like protease (PLpro).15,16 Therefore, understanding the functions of the relevant proteases is crucial for identifying and developing the specific antiviral drugs that can effectively prevent or treat COVID-19. The diverse range of proteases involved in COVID-19 infection not only presents significant challenges but also provides abundant potential opportunities to target proteases as an antiviral strategy (Figure 1).

In this Review, we discuss how diverse proteases participate in the pathogenesis of COVID-19 and present the protease inhibitors for COVID-19 treatment, which includes identifying the currently available FDA-approved drugs to be repurposed.
for COVID-19 treatment and suggesting possible crystal structures for developing new specific antivirus medicines, resulting from *in silico*, *in vitro*, or *in vivo* studies. Finally, we will summarize the progress of COVID-19 clinical studies for the protease inhibitors.

2. HOST PROTEASE

SARS-CoV-2 cell entry depends on ACE2 and a diverse range of host proteases. Host proteases prime the S protein of coronaviruses for high-affinity binding with ACE2 and efficient fusion with host lipid membranes to release viral materials. SARS-CoV-2 has evolved a variety of strategies for the proteolytic activation of the S protein, involving a large number of host proteases for proteolytic processes. These host proteases may include, but are not limited to, cell surface transmembrane protease/serine (TMPRSS) proteases, furin, cathepsins, plasmin, elastase, and trypsin. A recent study also showed that for SARS-CoV-2, but not SARS-CoV, the S proteins induced the formation of cell–cell fusion events (syncytia) on 293/hACE2 cells, even in the absence of trypsin, suggesting that SARS-CoV-2 S proteins, to a certain degree, could trigger membrane fusion upon the receptor binding even without exogenous protease priming or activation. The sophisticated cell entry mechanisms of SARS-CoV-2 pose significant challenges for an antiviral strategy targeting host proteases but also illuminate potential effective intervention strategies that target the cell entry of the virus. We would also need to consider side effects when these drugs target host proteases.

2.1. TMPRSS Protease Family

TMPRSS proteases are part of the larger family of membrane-bound serine proteases found on the plasma membrane or in the secretory pathway of cells. TMPRSS proteases are widely expressed in the nasopharynx, respiratory tract, intestinal tract, and so on, where they are involved in the tropism and pathogenesis of coronaviruses, influenza viruses, and other respiratory viruses. Among the members of the TMPRSS protease family, TMPRSS2 and TMPRSS4 have been recognized to play critical roles in SARS-CoV-2 activation and proliferation. SARS-CoV-2 infection via the ACE2 and TMPRSS families is by direct fusion with the plasma membrane and is independent of the endocytosis pathway. This implicated antiviral treatment targeting for the neutralization of the endosome/lysosome pH, for example, by chloroquine, hydroxychloroquine, or the direct inhibition of cathepsin by Ed64, will have much attenuated effects in TMPRSS+ cells. The recent failure of a series of chloroquine and hydroxychloroquine clinical trials potentially resulted in the recent failure of a series of chloroquine and hydroxychloroquine clinical trials.
from their failure to inhibit the mass production of viral particles in TMPRSS+ cells (e.g., nasal epithelia, pulmonary AT2 cells, intestine enterocytes, etc.).

**TMPRSS2.** TMPRSS2 is a transmembrane protease with no secreted form. It may also work intracellularly at the trans-Golgi network (TGN).\(^2^5\) TMPRSS2 expressed in the same cell as ACE2 receptor (\emph{in cis}) could be optimal for the activation of SARS-CoV-2, but it does not exclude potential \emph{in trans} activation. There have been several studies suggesting that ACE2 and TMPRSS2 coexpress at the single-cell level in isolated human primary cells and cell lines including alveolar epithelial type II cells,\(^2^6\) bronchial transient secretory cells, ileal absorptive enterocytes,\(^2^7\) nasal epithelium,\(^2^8\) primary conjunctival and pterygium cells derived from the human cornea,\(^2^9\) primary human umbilical vein endothelial cells (HUVECs),\(^2^9\) as well as prostate epithelial cells.\(^3^0\)

TMPRSS2 and ACE2 coexpression enables viral particles to fuse directly with the plasma membrane, bypassing the slow endocytic pathway and potential lysosomal degradation and ensuring that viruses infect host cells with high efficiency. We believe that TMPRSS2/ACE2-mediated viral entry and proliferation are central to the SARS-CoV high transmission rate and pathogenesis.

TMPRSS2 is known to mediate proteolytic cleavage at both the S1/S2 and S2 domains. S1/S2 cleavage releases the receptor binding domain (RBD) for high-affinity binding with ACE2, whereas S2 cleavage releases the S2 domain for efficient fusion with the plasma membrane.\(^3^1\) The SARS-CoV-2 RBD, albeit binding ACE2 with more avidity,\(^3^1\) is less exposed than the SARS-CoV RBD. The cryo-electron microscopy (cryo-EM) structure of the SARS-CoV-2 S protein found that its RBD is mostly in the lying-down state,\(^2^2,2^3\) a state associated with inefficient receptor binding, despite the fact that the RBD of SARS-CoV-2 binds the ACE2 receptor with greater affinity than that of SARS-CoV. TMPRSS2 could have helped to switch the RBD from a lying-down position for immune evasion to a standing-up position for receptor binding at the target cell.\(^1^3\) It is also possible that other relevant proteases such as furin matured the viral S protein at the S1/S2 domain during viral assembly and then released it for high-affinity binding with ACE2 receptors.\(^3^2,3^3\)

**TMPRSS4.** Both ACE2 and TMPRSS2 are highly expressed in the gastrointestinal (GI) tract.\(^3^4\) ACE2 expression is much higher in the small intestine than any other organs including the lung in both humans and mice, making the GI tract an ideal secondary habitat for SARS-CoV-2 proliferation following pulmonary infection. Gastrointestinal symptoms are frequently observed in COVID-19 patients,\(^3^5\) and fecal shedding of SARS-CoV-2 RNA could persist weeks after the lung infection has diminished,\(^3^6\) suggesting the potential immune-privileged enteric niches for SARS-CoV-2. However, a recent study showed that ACE2 was highly expressed in intestine enterocytes but not in goblet and endocrine cells, where TMPRSS2 was expressed.\(^3^7,3^8\) Instead, TMPRSS4 was coexpressed in ACE2+ mature enterocytes, whereas TMPRSS2 was primarily expressed in ACE2− secretory intestinal epithelial cells (IECs).\(^2^5\) The abrogation of TMPRSS4 expression by CRISPR led to a four-fold reduction in VSV-SARS-CoV-2 replication in human enteroids, greater than seen for TMPRSS2 knockout cells. This suggests that TMPRSS4 instead of TMPRSS2

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**Figure 2.** SARS-CoV-2 S-protein activation by host proteases. (A) Schematic of SARS-CoV-2 and (B) spike protein. (C) Sequence alignment of SARS-CoV, MERS-CoV, and SARS-CoV-2 in protease cleavage sites S1/S2 and S2. Only the SARS-CoV-2 spike contains a putative furin cleavage motif, RRAR (labeled in red). The receptor binding domain (RBD) within the S protein can be released by cleavage at the S1/S2 site by furin, TMPRSS2&4, and trypsin to generate the optimal conformation for ACE2 receptor binding. S2 domain cleavage by TMPRSS2&4 enabled the viral membrane fusion with the host membrane.
played major roles in mediating SARS-CoV-2 infection in the intestine.20

It is important to note that TMPRSS4 and TMPRSS2 could function in trans16 to preclude the S protein from the adjacent cells, especially in semienclosed folding structures like alveoli and intestinal glands. In addition, the proteolytic cleavage of the S protein by TMPRSS family members could occur in the TGN, where the virus particles would be preprimed with both high-affinity ACE2 binding as well as fusogenic propensity (Figure 1). In this scenario, infected ACE2+/TMPRSS+ cells would be a factory mass-producing the highly infectious and immunogenic “ready-to-kill” virions, which would result in a fast and wide-spreading viral infection and a drastic immune response across the body. Thus, if true, the control of viral production in hotspot cells (ACE2+/TMPRSS2+ and TMPRSS 4+) could prove to be critical for treatment, and these potential statioptemporal protease cleavage events are worth future investigations.

2.2. Furin Protease

Disturbingly, SARS-CoV-2 evolved to possess a furin cleavage site at its S1/S2 boundary site next to the predicted TMPRSS2 site (Figure 2C). Cleavage of the spike protein at the S1/S2 site by furin is essential for S-protein-mediated cell−cell fusion and entry into human lung cells. In our unpublished data, furin greatly enhanced the VSV-SARS-CoV-2-S protein pseudovirus infection capability when coexpressed with ACE2 receptor.

Furin proteases, a subtilisin-like peptidase encoded by the FURIN gene, cleave at paired basic amino residues. Furin is a membrane-bound protease that can also be shed in the extracellular space, is constitutively secreted, and is mainly expressed in epithelial cells of the skin, lung, kidney, and liver, as well as in neuronal cells. Furin is already in the stand-up position for high-affinity ACE2 binding as well as fusogenic propensity.25 (Figure 1). In this scenario, infected ACE2+/TMPRSS+ cells would be a factory mass-producing the highly infectious and immunogenic “ready-to-kill” virions, which would result in a fast and wide-spreading viral infection and a drastic immune response across the body. Thus, if true, the control of viral production in hotspot cells (ACE2+/TMPRSS2+ and TMPRSS 4+) could prove to be critical for treatment, and these potential statioptemporal protease cleavage events are worth future investigations.

2.3. Trypsin

Trypsin is a prototype serine endopeptidase found in the digestive system of many vertebrates, which cleaves at a neutral pH or a slightly basic pH of 8.0.27 Trypsin is also found to be expressed in epithelial cells of the skin, lung, kidney, and liver, and splenic and neuronal cells by in situ hybridization and immunohistochemistry. Biochemically, trypsin is less specific and strongly prefers to cleave at arginine (R) or lysine (K) residues. Furin has been extensively studied for virus glycoprotein cleavage activation, and it had been shown to activate SARS-CoV-2 in vitro.28 In fact, trypsin has been used as a surrogate for more biologically relevant proteases such as members of the TMPRSS family, which have similar substrate specificities (Figure 2C). Trypsin is expressed in the respiratory tract with lower expression, and its activity is balanced by α-1 antitrypsin.29 SARS-CoV is produced in VeroE6 cells, where trypsin can override the need for low-pH-dependent cathepsin-mediated cleavage in vitro and possibly shifts the virus to entry directly at the plasma membrane. Thus trypsin is likely to be able to directly cleave the S proteins in both the lung and small intestine as a supplement to TMPRSS and furin. The pharmacological inhibition of trypsin by the FDA-approved drug aprotinin via inhalation or injection could also help to mitigate SARS-CoV-2 infection in the lungs.18

2.4. Cathepsin Protease

Cathepsin proteases belongs to a family of cysteine proteases that are expressed almost exclusively in all mammalian lysosomes and plays an important role in intracellular proteolysis and viral entry.14 It is worth noting that TMPRSS was expressed in only a subset of ACE2+ cells; however, cathepsin B was promiscuously expressed in >70–90% of ACE2+ cells.28 In ACE2+/TMPRSS− cells, SARS-CoV-2 entry could be facilitated by ACE2 internalization. Upon vesicle internalization and acidification, the S protein is then cleaved by cathepsin B/L to release the virus contained by membrane fusion. Hydroxychloroquine and chloroquine could effectively neutralize the lysosomal pH and inhibit cathepsin in vitro.29 Presumably, hydroxychloroquine and chloroquine could still inhibit virus proliferation in ACE2+/TMPRSS− cells. However, whereas TMPRSS2 activity is documented to be important for viral transmission, the potential of cathepsin B/L or other proteases to functionally replace TMPRSS2 has not been determined.18 Indeed, chloroquine failed to inhibit SARS-CoV-2 pseudovirus infection in ACE2+/TMPRSS+ cells51 and the recent clinical trials on hydroxychloroquine have not yielded positive results for reducing mortality.50

2.5. Factor Xa Protease

Factor Xa is another protease that was shown to activate the SARS-CoV S protein for entry into host cells by cleavage at the S1/S2 boundary,51 although the involvement of factor Xa in SARS-CoV-2 activation has not yet been proven. Factor Xa also participates in the pathological airway remodeling during asthma and other pro-inflammatory diseases,52 suggesting beneficial effects by the inhibition of factor Xa for COVID-19 patients. Factor Xa protease is closely related to the blood
coagulation cascade in sepsis induced by viral infection. Pulmonary artery thrombosis, cardiovascular artery thrombosis, and disseminated intravascular coagulation (DIC) have been often found in patients with SARS and MERS as well as in COVID-19 patients. The prophylactic or treatment use of anticoagulants, including low-molecular-weight (LMW) heparin as well as factor Xa inhibitors, have been proved to be critical for reducing mortality in COVID-19 patients.

2.6. Plasmin

Plasmin is produced from its precursor plasminogen. Plasmin is a key enzyme in blood clot lysis, and its major natural substrates are fibrinogen and fibrin. Elevated plasmin(ogen) is a common feature in people who are susceptible to SARS-CoV-2 infection (e.g., hypertension, diabetes, cardiovascular disease, cerebrovascular disease, and chronic renal illness). Because plasmin has been shown to activate SARS-CoV S, it could potentially enhance the virulence and infectivity of the SARS-CoV-2 virus, which still needs to be proved. An extremely increased D-dimer in COVID-19 patients was observed as a consequence of plasmin-associated hyperactive fibrinolysis. D-dimer and viral load are independent risk factors of disease severity and mortality. However, D-dimer elevation is the marker and consequence of dramatically increased blood coagulation. DIC, rather than increased D-dimer due to plasmin-associated hyperactive fibrinolysis, caused increased mortality. Antiprotease measures targeting plasmin could artificially reduce the D-dimer by blocking fibrinolysis, which could actually aggravate the DIC symptoms in severe patients. Thus we would caution the use of antiproteases targeting plasmin as a promising treatment, as suggested in another review.

2.7. Elastase

Elastases are a family of proteases characterized by their ability to break down insoluble elastin fibers. The amino acid preferences for elastase are very different from those of trypsin; however, it has also been shown to activate the SARS-CoV S protein and facilitate SARS-CoV entry via a low-pH-independent route. The neutrophil elastase breaks down the structural proteins and virulence factors of invading bacteria. Besides its physiological function as a powerful host
defense, neutrophil elastase is also known as one of the most destructive enzymes in the body. Neutrophil activation and infiltration into the lung are characteristic of COVID-19 infection. Neutrophil elastase could have caused the acute lung injury and triggered the neutrophil extracellular traps (NETs) in COVID-19 to release DNA and proteins to form suffocating hyaline membranes lining the alveoli and to trigger DIC in blood vessels. Both pathological processes are highly prevalent in COVID-19 patients (Figure 3). Thus agents targeting neutrophil elastase release and activation will probably have multifaceted beneficial effects for combating COVID-19.

3. VIRAL PROTEASES

3.1. Main Protease

As in other coronaviruses, the main protease (Mpro) of the SARS-CoV-2 plays an important role in the viral maturation by processing many polyproteins that are translated from the viral RNA. It is well known that Mpro performs the cleavage on 12 nonstructural proteins (Nsp4-Nsp16), including critical proteins like the RNA-dependent RNA polymerase (RdRp, Nsp12) and helicase (Nsp13). Because of its vital activity for SARS-CoV-2, Mpro represents one of the most attractive antiviral drug targets. Indeed, several experimental studies have already demonstrated that the Mpro inhibition can prevent the virus from replication. However, so far, an FDA-approved Mpro inhibitor is still missing for the SARS-CoV-2 Mpro.

Several crystal structures of the SARS-CoV-2 Mpro with and without bound inhibitors have been recently obtained in experiments. Demonstrated in in vitro viral proliferation models, irreversible inhibitors like N3 (PDB code 6LU7), camofur (PDB code 7BUY), and α-ketoamide (PDB code 6Y2F) are efficacious at inhibiting the SARS-CoV-2 virus with moderate affinities (EC50: 1–20 μM) but with the benefit of k_{off} being close to zero. Furthermore, regular noncovalent bound drugs such as baicalein (PDB code 6M2N; EC50 ≈ 1.7 μM) and X11 (PDB code 6W63) can be bound inside the Mpro’s pocket with hydrophilic hydrogen bonds and hydrophobic interactions, as captured in crystal structures. However, except for camofur (a former FDA-approved drug for breast cancer that is currently withdrawn due to the safety issue) and baicalein (a herbal medicine), the development of these tool drugs into an FDA-approved drug could take years to accomplish.

With the evidence obtained from various experiments supporting the notion that Mpro can be a viable antiviral target, the research efforts to find more potent and specific antiviral drugs based on this target are warranted. Nowadays, using the world’s most powerful supercomputer such as the IBM POWER9-based Summit, or a high-performance computing cluster, it is possible to perform very large-scale virtual screening on drug molecules from large databases (e.g., ZINC15) to identify the most potent candidate inhibitors for Mpro. For example, two available drugs (talampicillin and lurasideone) and two drug-like compounds (ZINC000000702323 and ZINC000012481889) were recently discovered in silico for inhibiting SARS-CoV-2 Mpro. The anti-HIV drugs lopinavir and ritonavir were shown in silico to bind well with residues at the active site of SARS-CoV-2 Mpro. Additionally, natural compounds such as quercetin and rutin were also found to inhibit the SARS-CoV-2 Mpro in silico.

Besides high-throughput screening, in silico drug repurposing aimed at the fast discovery of antiviral drugs in the current pandemic has provided an alternative efficient way to search for potent drugs to fight COVID-19. For example, on the basis of a small list of approved drugs previously applied for the treatment of SARS-CoV/MERS and the ones that are under current clinical trials for SARS-CoV-2, an in silico approach combining both docking and all-atom molecular dynamics simulations was successfully employed to investigate the underlying molecular mechanism of the drugs’ binding inside the Mpro’s pocket (Figure 4), revealing an important phenomenon in which high-potent drugs (such as nelfinavir) generally occupy the so-called “anchor” site in the Mpro’s pocket. The following in vitro experiment confirmed that
nellinavir can inhibit SARS-CoV-2 Mpro with an IC50 of \(~0.77\ \mu M\).\(^7^5\)

Both experimental and in silico efforts provide an invaluable understanding of the structural determinants for ligand–Mpro binding, which is critical for the future design and optimization of inhibitors for the SARS-CoV-2’s Mpro. Moreover, a similar approach can be applied to search inhibitors for other viral proteases as well as human proteases, such as TMPRSS2 and furin, that are crucial for the entry of SARS-CoV-2 into host cells.

3.2. Papain-like Protease
PLpro is responsible for processing nonstructural proteins Nsp1, Nsp2, and Nsp3, which are released after the cleavage of the N-terminus of the replicase polyprotein, and thus is crucial for viral replication. Experimental research on PLpro is still in its early stages, and hence there are only a few crystal structures available in the Protein Data Bank, with their corresponding papers to be published. So far, there are two apo crystal structures (PDB codes 6W9C and 6WZU) and another two with bound irreversible compounds VIR250 (PDB code 6WUU) and VIR251 (PDB code 6WX4), indicating that PLpro potentially can be a viable target for drugs.

Despite the lack of a more complete understanding of the existing crystal structures, in silico studies have identified a few possible drug molecules targeting PLpro. For example, by screening the FDA-approved drugs, it was found that bilirubin can bind PLpro efficaciously.\(^7^6\) In a docking study, darunavir was revealed to have a strong binding with PLpro.\(^7^7\) Likewise, using the docking method, inarigivir from a database of antiviral agents was found to inhibit both the PLpro and Mpro simultaneously.\(^7^8\) Overall, compared with Mpro, only a limited amount of research works have been focused on PLpro. However, with newly available crystal structures, there are expected to be more in silico studies as well as in vitro and in vivo experiments based on this protease in the near future.

4. PROTEASE INHIBITORS IN COVID-19-RELATED CLINICAL STUDIES
COVID-19 has become a global threat to public health and has been impacting society in nearly all aspects since early 2020. To combat COVID-19, there has been a concentrated effort to develop drugs and vaccines to treat COVID-19 patients and to immunize the public to eliminate the epidemic spreading. Despite the fact that, on average, it takes more than 12 years to bring a new drug from preclinical discovery to patients, clinical trials started shortly after the first onset of the outbreak, focusing on repurposing drugs and compounds that were originally developed for other diseases, such as ebola, HIV, and influenza, to treat COVID-19 patients.\(^7^9\) As of May 29, 2020, about 4 months after the initial outbreak in China, there have been 1833 COVID-19-related clinical studies reported to clinicaltrials.gov, and the number is still rapidly rising. Such surging efforts reflect the urgent need to find effective medical solutions to cease this pandemic.

Among the 1833 reported clinical studies, 826 of them involve drug or biological interventions\(^8^1\) (queried on May 29, 2020). Attention has been devoted to demonstrating the safety of the investigated compounds, validating the efficacy of the drug candidates on COVID-19 patients, as well as quantifying the differences in the disease progression rate and the infection rate within various subpopulations, defined by factors such as pre-existing medical conditions, ongoing medical interventions, and preventive medications. From a pathologic standpoint, it is evident that both the host and the viral proteases (e.g., Mpro, PLpro, etc.) play critical roles in the various stages of SARS-CoV-2 infection as well as the COVID-19 disease progression.\(^1^4\)–\(^1^6\) Therefore, in regards to protease inhibitor (PI) drugs for COVID-19 treatment, at least two noticeable types of hypotheses exist: On the one hand, PI drugs that have been used to treat other viral infections are considered as potential candidates for treating COVID-19 patients with previously demonstrated drug safety. On the other hand, the population that is experiencing immune deficiency but is also under antiviral therapies may have a different infection rate when exposed to SARS-CoV-2 or may have a different risk of progressing to the severe stage after infection.

Back to the 2003 SARS outbreak, Chan and coworkers reported that adding lopinavir–ritonavir as an initial treatment to the standard care protocol was associated with a reduction in the overall death rate and intubation rate.\(^8^2\) Therefore, lopinavir–ritonavir, both protease inhibitors used for HIV treatment and prevention,\(^8^3\) were among the earliest drug candidates to treat COVID-19 patients.\(^8^4\) However, in a randomized, controlled, open-label trial targeting adults with severe illness caused by SARS-CoV-2, Cao and colleagues reported that there were no significant differences in the time to clinical improvement or mortality at 28 days between the lopinavir–ritonavir treatment and the standard care groups.\(^8^5\) This set of results suggested that lopinavir–ritonavir may have only a limited role in treating severely ill COVID-19 patients.\(^3\) Follow-up studies may consider using lopinavir–ritonavir to treat mild or moderate COVID-19 patients, for example, as part of the initial treatment.

Besides lopinavir–ritonavir, many other protease inhibitor drugs are also being investigated, and among the 826 drug/biologic-related studies reported to clinicaltrials.gov, close to 100 trials (\(~10\%) involve one or multiple protease inhibitor drugs, including favipiravir and oseltamivir, which are used for influenza, darunavir, which is used for HIV, and danoprevir, which is used for hepatitis C infection\(^8^1\) (queried on May 29, 2020). There have been accumulated preliminary reports demonstrating the potential effects of these PI drugs on COVID-19 patients.\(^5^6\)–\(^5^8\) With more clinical trials reaching their study end points, we may establish more effective strategies for using PI antiviral drugs, with other medications, in COVID-19 treatment.

In addition to the interventional studies, there are at least seven observational studies specifically targeting the population with immune deficiencies, including HIV patients, and aiming to understand the impacts of COVID-19 on this patient group. An early study by Zhu et al. reported the coinfected case of SARS-CoV-2 and HIV and suggested that “HIV patients should be regarded as vulnerable group”.\(^8^9\) However, this reported patient did not have previous antiviral therapy, which is less typical.\(^9^0\) A more recent study by Härter et al. investigated a retrospective and uncontrolled case series involving 33 HIV patients and found that symptomatic COVID-19 and HIV coinfected patients with viral suppression on ART do not exhibit higher morbidity and mortality compared with other patients.\(^9^1\) This study also indicated that SARS-CoV-2 infections may still occur during darunavir-based treatment. Because of the limitations of these case studies, more rigorously designed clinical studies with larger patient cohorts and proper control groups are needed to demonstrate...
| market status | potential candidate | target | IC₅₀ (µM) | mechanism |
|---------------|---------------------|--------|-----------|-----------|
| natural product | baicalein (PDB code 6M2N) | Mpro | 0.945 (by enzymatic assay) | Baicalein binds to the substrate pocket by interacting with two catalytic residues to prevent the peptide substrate from approaching the active site.\(^{66}\) |
| approved | talampicillin | Mpro | 12.01 \(^{65}\) (by cell-based assay) | Talampicillin and lurasidone showed a reliable binding pattern in Mpro and closed the active site of the enzyme.\(^{70}\) |
| approved | lopinavir | Mpro | 12.01 \(^{65}\) (by cell-based assay) | Lopinavir interacts with the active site through hydrogen-bond formation with Arg911 and hydrophobic interaction with Tyr 1013 to inhibit Mpro.\(^{16,71,78}\) |
| approved | ritonavir | Mpro | 19.88 \(^{65}\) (by cell-based assay) | The drug binds to the surrounding residues in the active site of SARS-CoV-2 3CLpro and inhibits Mpro.\(^{16,71}\) |
| approved | neliniavir | Mpro/ PLpro | 0.77 \(^{73}\) (by cell-based assay) | The benzamide carbonyl group and octahydro-1H-isoquinoline moiety interact with Gly143 through a H bond and Glu166 by forming a H bond and salt bridge interaction. It can inhibit virus replication by combining with Mpro, and in combination with cepharanthine, it can inhibit the proliferation of SARS-COV-2.\(^{75,78}\) |
| approved | valgnociclovir | Mpro/ PLpro | Virtual binding shows that it can bind to Mpro and PLpro, so it may be a dual-enzyme inhibitor.\(^{71}\) |
| approved | inariavir | Mpro/ PLpro | Using the docking method, it can bind to PLpro and Mpro, so it may have double-enzyme inhibition.\(^{78}\) |
| approved | camostat (Foipan) | TMPRSS2 | 6.2 nM\(^{65}\) (by protein enzymatic assay) | Camostat is a clinically proven commercially synthesized serine protease inhibitor. The inhibition of TMPRSS2 by camostat can significantly reduce the infection of SARS-CoV-2.\(^{19,94,95}\) |
| approved | nafamostat (Buipel) | TMPRSS2 | 0.27 nM\(^{65}\) (by protein enzymatic assay) | Nafamostat is a synthetic serine protease inhibitor approved by Japan. By inhibiting TMPRSS2, it inhibits the activation of SARS-CoV-2 S protein, thus inhibiting the infection of SARS-CoV-2 on human lung cells.\(^{94,96}\) |
| approved | bromhexine | TMPRSS2 | 0.75µM \(^{73}\) (by protein enzymatic assay) | The metastasis inhibitory factor of prostate cancer was found by chemical library screening, which confirmed that bromhexine is an effective selective inhibitor of TMPRSS2.\(^{98-100}\) |
| tool compound | N3 (PDB code 6LU7) | Mpro | 16.8 µM (by cell-based assay) | The inhibitor first binds to SARS-CoV-2 Mpro; then, a stable covalent bond is formed between Mpro and N3. N3 forms multiple hydrogen bonds with the main chain of the residues in the substrate-binding pocket.\(^{66}\) |
| approved | camofur (PDB code 7BUY) | Mpro | 24.3 \(^{65}\) (by cell-based assay) | By high-throughput screening, carmofur is able to covalently bind to C145 of the catalytic dyad in SARS-CoV-2 Mpro.\(^{66,67}\) |
| approved | ebselen | Mpro | 0.67 \(^{65}\) (by protein enzymatic assay) | Ebselen has the strongest inhibition of Mpro activity with an IC50 of 0.67 µM. Ebselen may inhibit Mpro through noncovalent binding.\(^{66}\) (high-throughput screening) |
| tool compound | α-ketoamide (PDB code 6Y2F) | Mpro | 0.67 ± 0.18 \(^{65}\) (by cell-based assay) | α-Ketoamide is a designed and synthesized Mpro inhibitor, which can inhibit the action of Mpro by interacting with the catalytic center of the target protease through two hydrogen bonds.\(^{65}\) |
| approved | adafosbuvir | Mpro | | The compound formed hydrogen bonds with Gly143 and Gln189 main-chain amines and accumulated with His41. These amino acids existed in Mpro, thus inhibiting the effect of Mpro on SARS-CoV-2.\(^{65}\) (virtual ligand screening) |
| approved | elusilavirine | PLpro | | Elusilavirine interacts with the PLpro substrate binding site by H-bond formation with Asp909, Gln1014, and Tyr1018 as well as hydrophobic and electrostatic interactions with Tyr1013 and Lys902, respectively.\(^{71}\) (virtual ligand screening) |
| approved | maribavir | PLpro | | Maribavir is an investigation compound for use/treatment in viral infection that interacts with Asp909, Gln1014, Tyr1018, and Tyr1013 through H-bond formation and hydrophobic interactions. These amino acids are involved in the formation of PLpro active sites.\(^{71}\) |
| natural product | quercetin | Mpro | | Quercetin is able to form complexes with the Mpro with good binding affinities by molecular dynamics (MD) simulations.\(^{73}\) |
| natural product | danoprevir | Mpro | | The relevant articles on its mechanism of inhibiting SARS-CoV-2 have not yet been found. |
| natural product | rutin | Mpro | | The relevant articles on its mechanism of inhibiting SARS-CoV-2 have not yet been found. |
the vulnerability of HIV patients to SARS-CoV-2 and the effect of PI antiviral therapies on protecting this population.

We summarize the marketed drugs and some tool drugs that are potentially useful for clinical practice and research purposes in Table 1.

5. CONCLUSIONS

Proteases are promising drug targets for the antiviral treatment of COVID-19, but the drug development and therapeutics toward them could be a very complicated process. We have to take into account the efficacy and toxicity profile of protease modulators at the enzymatic, cellular, organ, as well as system levels. Deeply understanding the complexity of viral–host interactions in terms of proteases is critical for developing effective yet low-toxicity treatments and preventive therapies for wide-spreading diseases like COVID-19. The careful selection of one or multiple protease targets and the method with which we apply the modulators in different stages of the disease are crucial for successfully engaging proteases. For example, in the early stage, we recommend the use of officially approved drugs such as camostat and nafamostat via inhalation (6) Zhou, P.; et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 2020, 579, 270–273.

(7) McMichael, T. M.; et al. Epidemiology of Covid-19 in a Long-Term Care Facility in King County, Washington. N. Engl. J. Med. 2020, 382, 2005–2011.

(8) Zheng, S.; et al. Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January-March 2020: retrospective cohort study. BMJ, 2020, m1443.

(9) Neuman, B. W.; et al. Supramolecular Architecture of Severe Acute Respiratory Syndrome Coronavirus Revealed by Electron Cryomicroscopy. J. Virol. 2006, 80, 7918–7928.

(10) Nature 1968, 220, 650.

(11) Simmons, G.; Zmora, P.; Gierer, S.; Heurich, A.; Pöhlimann, S. Proteolytic activation of the SARS-coronavirus spike protein: Cutting enzymes at the cutting edge of antiviral research. Antiviral Res. 2013, 100, 605–614.

(12) Fehr, A. R.; Perlman, S.; Maier, H. J.; Bickerton, E.; Britton, P. Coronaviruses: An Overview of Their Replication and Pathogenesis. Coronaviruses 2015, 1282, 1–23.

(13) Shang, J.; et al. Cell entry mechanisms of SARS-CoV-2. Proc. Natl. Acad. Sci. U. S. A. 2020, 117, 11727–11734.

(14) Millet, J. K.; Whittaker, G. R. Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis. Virus Res. 2015, 202, 120–134.

(15) Anand, K. Coronavirus Main Protease (3Clpro): Structure Basis for Design of Anti-SARS Drugs. Science 2003, 300, 1763–1767.

(16) Lin, S.; Shen, R.; He, J.; Li, X.; Guo, X. Molecular Modeling Evaluation of the Binding Effect of Ritonavir, Lopinavir and Darunavir to Severe Acute Respiratory Syndrome Coronavirus 2 Proteases. bioRxiv 2020, DOI: 10.1101/2020.01.31.929695.

(17) Tang, T.; Bidon, M.; Jaimes, J. A.; Whittaker, G. R.; Daniel, S. Coronavirus membrane fusion mechanism offers a potential target for antiviral development. Antiviral Res. 2020, 178, 104792.

(18) Ou, X.; Liu, Y.; Lei, X.; Li, P.; Mi, D.; Ren, L.; Guo, L.; Guo, R.; Chen, T.; Hu, J. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. Nat. Commun. 2020, 11, 1620.

(19) Hoffmann, M.; Kleine-Weber, H.; Schroeder, S.; Kruger, N.; Herrler, T.; Erichsen, S.; Schiergens, T. S.; Herrler, G.; Wu, N.-H.; Nitsche, A.; et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell 2020, 181, 271–280.e8.

(20) Zhang, R.; et al. TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. Sci. Immunol. 2020, 5, eabc3582.

(21) Si, L.; et al. Human organs-on-chips as tools for repurposing approved drugs as potential influenza and COVID19 therapeutics in viral pandemics. bioRxiv 2020, DOI: 10.1101/2020.04.13.039917.

(22) Funnell, S. G. P.; Dowling, W. E.; Munoz-Fontela, C.; Grell, P.-S.; Ingber, D. E.; Hamilton, G. A.; Delang, L.; Rocha-Pereira, J.; Kaptein, S.; Dallmeier, K. H.; et al. Emerging preclinical evidence does not support broad use of hydroxychloroquine in COVID-19 patients. Nat. Commun. 2020, 11, 4253.

(23) Hoffmann, M.; et al. Chloroquine does not inhibit infection of human lung cells with SARS-CoV-2. Nature 2020, 585, 588–590.

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Coronavirus Disease 2019 in Wuhan, China. *JAMA Netw. Open* 2020, 3, No. e2010182.
(46) Guan, W.; et al. Clinical Characteristics of Coronavirus Disease 2019 in China. *N. Engl. J. Med.* 2020, 382, 1708–1720.
(47) Koshikawa, N.; et al. Expression of Trypsin by Epithelial Cells of Various Tissues, Leukocytes, and Neurons in Human and Mouse. *Am. J. Pathol.* 1998, 153, 937–944.
(48) Bojkova, D.; et al. SARS-CoV-2 and SARS-CoV differ in their cell tropism and drug sensitivity profiles. *bioRxiv* 2020, DOI: 10.1101/2020.04.03.024257.
(49) Wang, M.; et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* 2020, 30, 269–271.
(50) Mehra, M. R.; Desai, S. S.; Ruschitzka, F.; Patel, A. N. Hydroxychloroquine or chloroquine with or without a macrolide for treatment of COVID-19: a multinational registry analysis. *Lancet 2020*, DOI: 10.1016/S0140-6736(20)31180-6.
(51) Du, L.; et al. Cleavage of spike protein of SARS coronavirus by protease factor Xa is associated with viral infectivity. *Biochem. Biophys. Res. Commun.* 2007, 359, 174–179.
(52) Shinagawa, K.; Martin, J. A.; Ploplis, V. A.; Castellino, F. J. Coagulation Factor Xa Modulates Airway Remodeling in a Murine Model of Asthma. *Am. J. Respir. Crit. Care Med.* 2007, 175, 136–143.
(53) Riewald, M.; RUF, W. Science review: Role of coagulation protease cascades in sepsis. *Crit Care 2003*, 7, 123.
(54) Ng, K. H. L. Pulmonary artery thrombosis in a patient with severe acute respiratory syndrome. *Postgrad. Med. J.* 2005, 81, No. e3.
(55) Giannis, D.; Ziogas, I. A.; Gianni, P. Coagulation disorders in coronavirus infected patients: COVID-19, MERS-CoV, and lessons from the past. *J. Clin. Virol. 2020*, 127, 104362.
(56) Tang, N.; et al. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J. Thromb. Haemostasis 2020*, 18, 1094–1099.
(57) Perna, A. F.; et al. COVID-19, Low-Molecular-Weight Heparin, and Hemodialysis. *Kidney Blood Pressure Res.* 2020, 45, 357–362.
(58) Ji, H.-L.; Zhao, R.; Matalon, S.; Matthay, M. A. Elevated Plsaminogen(ogen) as a Common Risk Factor for COVID-19 Susceptibility. *Physiol. Rev. 2020*, 100, 1065–1075.
(59) Kam, Y.-W.; et al. Cleavage of the SARS Coronavirus Spike Glycoprotein by Airway Proteases Enhances Virus Entry into Human Bronchial Epithelial Cells In Vitro. *PLoS One 2009*, 4, No. e7870.
(60) Belouzard, S.; Madu, I.; Whitaker, G. R. Elastase-mediated Activation of the Severe Acute Respiratory Syndrome Coronavirus Spike Protein at Discrete Sites within the S2 Domain. *J. Biol. Chem.* 2010, 285, 22758–22763.
(61) Matsuyma, S.; Ujike, M.; Morikawa, S.; Tashiro, M.; Taguchi, F. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. *Proc. Natl. Acad. Sci. U. S. A.* 2005, 102, 12543–12547.
(62) Yan, X.; et al. Neutrophil to Lymphocyte Ratio as Prognostic and Predictive Factor in Patients with Coronavirus Disease 2019: A Retrospective Cross-sectional Study. *J. Med. Virol. 2020*, 92, 2573.
(63) Zuo, Y.; et al. Neutrophil extracellular traps in COVID-19. *JCI Insight 2020*, DOI: 10.1172/jci.insight.138999.
(64) Fox, S. E.; et al. Pulmonary and cardiac pathology in African American patients with COVID-19: an autopsy series from New Orleans. *Lancet Respir. Med.* 2020, 8, 681.
(65) Zhang, L.; et al. Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors. *Science 2020*, No. eabb3405.
(66) Jin, Z.; et al. Structure of Mpro from SARS-CoV-2 and discovery of its inhibitors. *Nature 2020*, 582, 289.
(67) Jin, Z.; et al. Structural basis for the inhibition of SARS-CoV-2 main protease by antineoplastic drug carmofur. *Nat. Struct. Mol. Biol.* 2020, 27, 529.
(68) Su, H. Discovery of baicalin and baicalein as novel, natural product inhibitors of SARS-CoV-2. *JCL protease in vitro. bioRxiv 2020*, DOI: 10.1101/2020.04.13.038687v1.
