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Reviews

Targeting Proteases for Treating COVID-19

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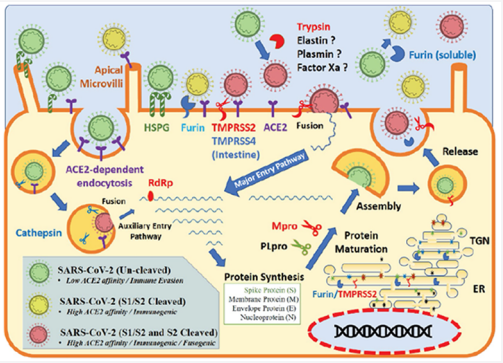
ABSTRACT: The unprecedented pandemic of coronavirus disease 2019 (COVID-19) demands effective treatment for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The infection of SARS-CoV-2 critically depends on diverse viral or host proteases, which mediate viral entry, viral protein 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.[3,5−8]

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

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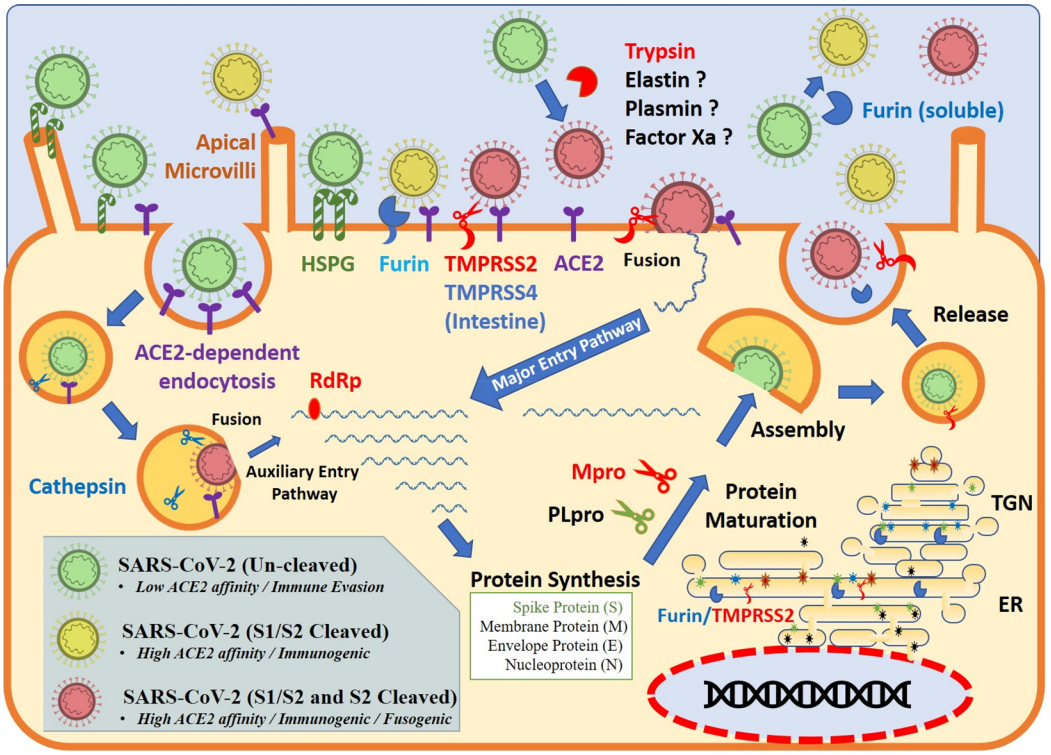

Figure 1. SARS-CoV-2 infection and proliferation process mediated by host and viral proteases. Diagram of the SARS-CoV-2 life cycle and the various host viral proteases known to activate some coronavirus S proteins. Coronaviruses and a variety of viruses bind to heparan sulfate proteoglycans (HSPGs) nonspecifically to initiate virus infection. With membrane proteases like TMPRSS2&4 and possibly furin, viral fusion could occur directly at the plasma membrane with high efficiency. pH-Dependent/cathepsin-mediated viral entry is also bypassed by endocytosis, which may only serve as an auxiliary entry pathway in TMPRSS+ cells. Extracellular proteases such as typsin, elastin, and factor Xa protease may also participate in the activation of the virus. Viral proteases Mpro and PLpro mediate the maturation of viral proteins (S, M, E, N) in the endoplasmic reticulum (ER), trans-Golgi network (TGN), and cytosol, which later package into virus particles. Furin, and possibly TMPRSS protease, could cleave the S protein in the ER and TGN system and the mature virion before it is released. We propose that there may be three models of SARS-CoV-2 particles: unactivated virion (uncleaved, green), semiactivated virion (S1/S2 cleaved, yellow), and fully activated virion (S1/S2 and S1 cleaved, red). ACE2+/furin+ and TMPRSS+ cells could serve as a factory of semiactive virion (yellow) and full-active virion (red) for the *in trans* infection of neighboring ACE2+ cells with or without membrane proteases. Notably, furin and cathepsin may be also released as soluble proteases and activate the virus extracellularly.

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.[13] 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.[17,14] 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.[18] 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 proteins.

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.[14] 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.[19,20] 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.[21] The recent failure of a series of chloroquine and hydroxychloroquine clinical trials[22−24] potentially resulted

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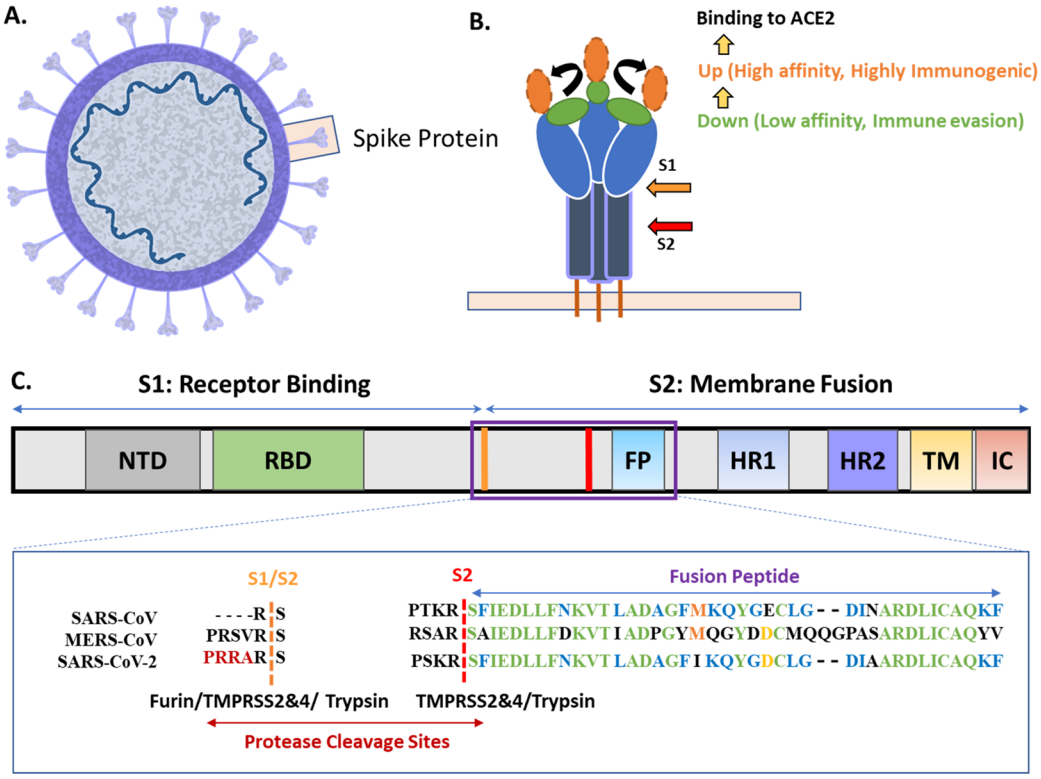

Figure 2. SARS-CoV-2 S-protein activation by host proteases. (A) Schematic of SARS-CoV-2 and (B) spike protein. (C) Sequence alignment of SARS-CoV, MERS-CoV, and SARS-CoV-2 in protease cleavage sites S1/S2 and S2. Only the SARS-CoV-2 spike contains a putative furin cleavage motif, RRAR (labeled in red). 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.

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).[25] TMPRSS2 expressed in the same cell as ACE2 receptor (*in cis*) could be optimal for the activation of SARS-CoV-2, but it does not exclude potential *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,[26] bronchial transient secretory cells, ileal absorptive enterocytes,[27] nasal epithelium,[28] primary conjunctival and pterygium cells derived from the human cornea,[29] primary human umbilical vein endothelial cells (HUVECs),[29] as well as prostate epithelial cells.[30]

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.[19] The SARS-CoV-2 RBD, albeit binding ACE2 with more avidity,[31] 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,[32,33] 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.[13] 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.[32,33]

TMPRSS4. Both ACE2 and TMPRSS2 are highly expressed in the gastrointestinal (GI) tract.[34] 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,[20] and fecal shedding of SARS-CoV-2 RNA could persist weeks after the lung infection has diminished,[35] suggesting the potential immune-privileged enteric niches[36] 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.[20,37] Instead, TMPRSS4 was coexpressed in ACE2+ mature enterocytes, whereas TMPRSS2 was primarily expressed in ACE2− secretory intestinal epithelial cells (IECs).[20] 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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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 trans*[38] to precleave 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[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 spatiotemporal 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.[39] 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 found in the TGN, where it is activated and can traffic to the plasma membrane. Furin may also recycle back from the plasma membrane to the TGN by endocytosis.[40] Furin is ubiquitously expressed; however it should be noted that in most cases, the levels of furin expression are low.[14,41] However, furin expression is enriched in the oral-pharyngeal cavity and upper respiratory tracts,[42] where the mucous protection is relatively weak.[43] Thus the virus could easily take advantage of furin in the oral-pharyngeal cavity for the proteolytic activation of the S protein and efficient infection/proliferation, as the seasonal flu virus does. Oral symptoms, such as dry mouth and taste blindness, are reported initial symptoms of COVID-19 infection.[44] Potential impairment of oral tissues should be also evaluated for insight into the prevention and treatment.

Different from SARS-CoV, whose highly immunogenic RBD is already in the stand-up position for high-affinity binding with the ACE2 receptor and immune recognition, the RBD of the SARS-CoV-2 S protein is less accessible until proteolytic cleavage by furin or TMPRSS2 at the S1/S2 domain,[13,32,33] which helps the virus to evade host immune surveillance during the dormant phase (Figure 2B). Obviously, this immune evasion strategy has been very successful because many COVID-19-infected patients are asymptomatic throughout the infection,[45] and there is also an extended asymptomatic period (up to several weeks) for later symptomatic patients,[46] whereas SARS or Middle East respiratory syndrome (MERS) patients almost immediately exhibited a high fever and other immune responses after infection, making the identification/isolation of infected individuals much more feasible. Host protease activation by furin could be a critical determinant of coronavirus infection and pathogenesis and a significant target for host immune surveillance and human intervention strategies. Therefore, we believe that furin protease-mediated viral activation could be the centerpiece of the sophisticated immune evasion strategy by SARS-CoV-2. Treatment targeting furin protease-mediated early infection events could prove to be critical for the containment of the infection.

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.[47] 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.[47] Biochemically, trypsin is less specific and strongly prefers to cleave at arginine (R) or lysine (K) residues. Trypsin has been extensively studied for virus glycoprotein cleavage activation, and it had been shown to activate SARS-CoV-2 *in vitro*.[18] 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.[14] 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.[48]

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. Hydroxylchloroquine and chloroquine could effectively neutralize the lysosomal pH and inhibit cathepsin *in vitro*.[49] Presumably, hydroxylchloroquine and chloroquine could still inhibit virus proliferation in ACE2+/TMPRSS2− 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.[28] Indeed, chloroquine failed to inhibit SARS-CoV-2 pseudovirus infection in ACE2+/TMPRSS+ cells,[21] and the recent clinical trials on hydroxylchloroquine 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

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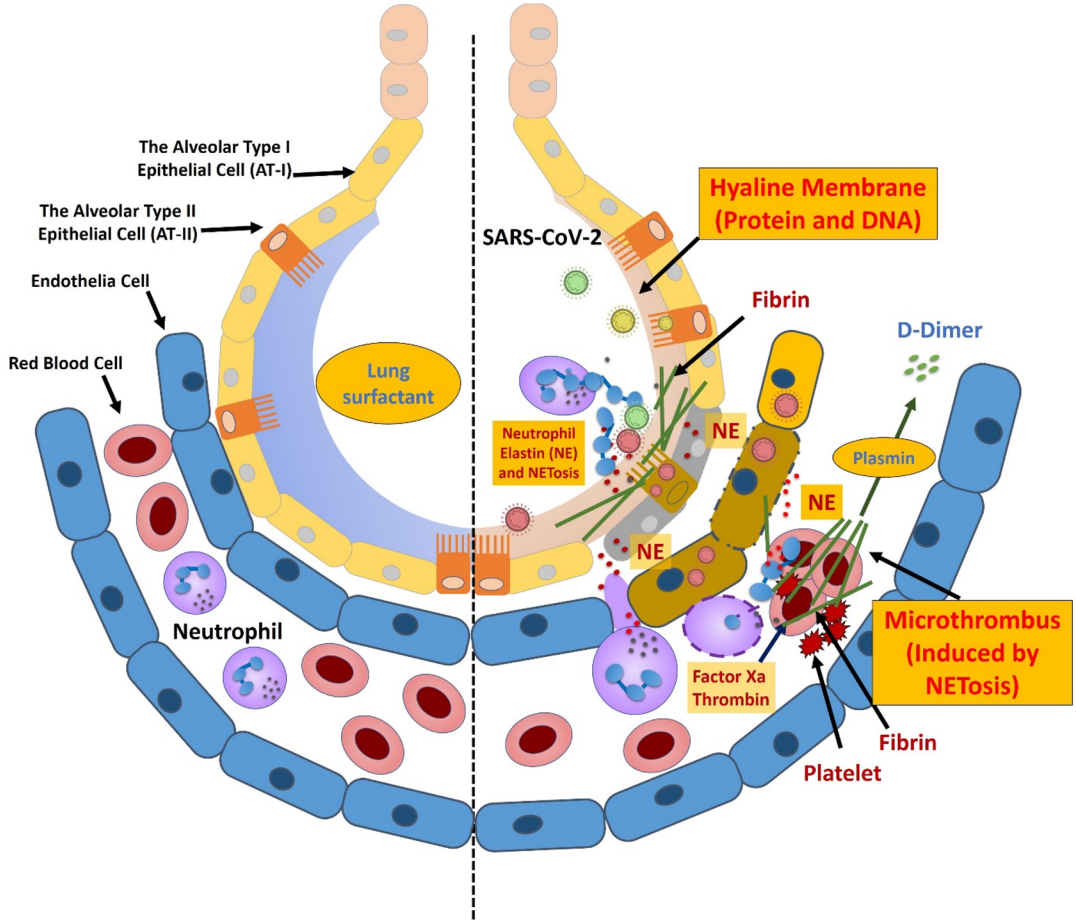

Figure 3. Pulmonary pathogenesis COVID-19 mediated by proteases. The alveolar epithelium is a continuous layer of epithelial cells consisting of alveolar type I (ATI) cells (thin cells that permit gas exchange) and alveolar type II (ATII) cells (which produce surfactant and antimicrobe protein/peptides to enable lung expansion with a low surface tension and prevent pathogen invasion). In SARS-CoV-2 pulmonary pathogenesis, the viral infection concentrated in ATII cells and reduced the surfactant production, which allowed further infections. Damaged epithelial cells increased alveolar−capillary permeability to fluid, proteins, neutrophils, and red blood cells, resulting in their accumulation in the alveolar space and the formation of hyaline membrane, a hallmark of acute respiratory distress syndrome (ARDS). Neutrophil secretion of neutrophil elastin (NE) protease damaged the tissue barriers and vascular endothelium cells, which triggered the fibrin formation and the release of the neutrophil extracellular trap (NETosis). NETosis together with fibrin further mobilized thrombin and factor Xa proteases, which then induced massive microthrombus in COVID-19 patients. Thus host proteases are active drivers for all three pulmonary pathogenesis hallmarks of COVID-19: neutrophil mobilization, hyaline membrane formation, and thrombus.

coagulation cascade in sepsis induced by viral infection.[53] Pulmonary artery thrombosis, cardiovascular artery thrombosis, and disseminated intravascular coagulation (DIC) have been often found in patients with SARS[54] and MERS[55] as well as in COVID-19 patients.[55] 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.[56,57]

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.[14] 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).[58] Because plasmin has been shown to activate SARS-CoV S,[59] 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.[58] 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.[58]

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.[60,61] The neutrophil elastase breaks down the structural proteins and virulence factors of invading bacteria. Besides its physiological function as a powerful host

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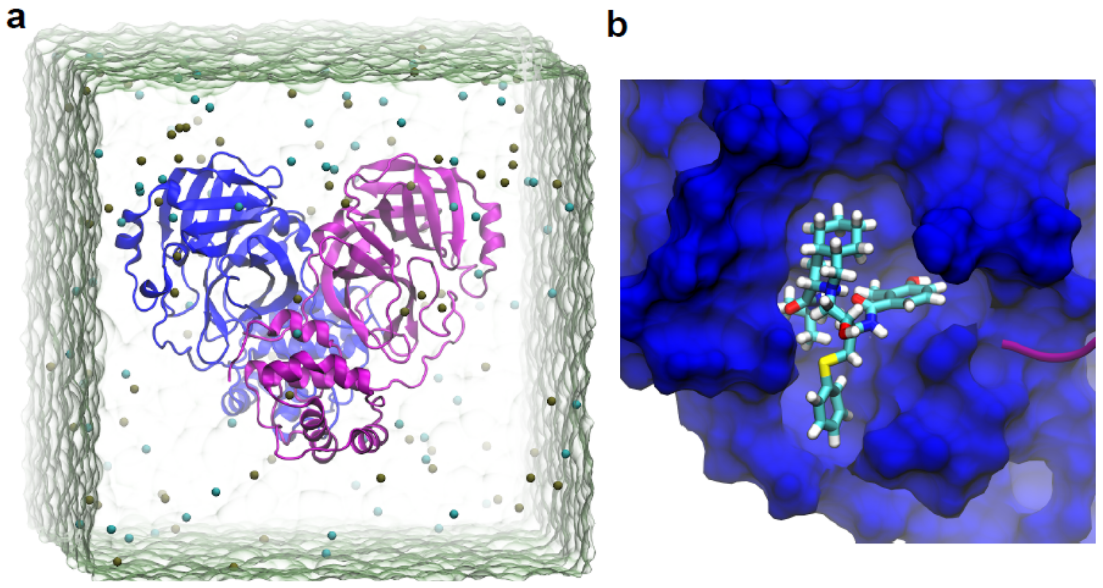

Figure 4. Molecular dynamics simulation for the SARS-CoV-2's Mpro. (a) Simulation system: Two monomers in the Mpro dimer (PDB: 6LU7) are in a cartoon representation and colored in blue and purple, respectively; K^+ and Cl^- ions are shown as van der Waals spheres and are colored in tan and cyan, respectively. Water is shown as transparent. (b) Nelfinavir inside the Mpro pocket. Adapted from the ref 74.

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.[62] Neutrophil elastase could have caused the acute lung injury and triggered the neutrophil extracellular traps (NETs) in COVID-19[63] to release DNA and proteins to form suffocating hyaline membranes lining the alveoli and to trigger DIC in blood vessels.[64] 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 SRAS-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.[15,65] 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),[66] camofur (PDB code 7BUY),[67] and α-ketoamide (PDB code 6Y2F)[65] 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 $\approx$ 1.7 μM)[68] 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.[69] For example, two available drugs (talampicillin and lurasidone) and two drug-like compounds (ZINC000000702323 and ZINC000012481889) were recently discovered *in silico* for inhibiting SARS-CoV-2 Mpro.[70] 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.[71] Additionally, natural compounds such as quercetin[72] and rutin[73] 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.[74] The following *in vitro* experiment confirmed that

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nelfinavir can inhibit SARS-CoV-2 Mpro with an IC50 of ~0.77 μM.[75]

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 biltricide can bind PLpro efficaciously.[76] In a docking study, darunavir was revealed to have a strong binding with PLpro.[77] Likewise, using the docking method, inarigivir from a database of antiviral agents was found to inhibit both the PLpro and Mpro simultaneously.[78] 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,[79] 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.[80] 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[81] (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.[14−16] 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.[82] Therefore, lopinavir−ritonavir, both protease inhibitors used for HIV treatment and prevention,[83] were among the earliest drug candidates to treat COVID-19 patients.[84] 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.[85] 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[81] (queried on May 29, 2020). There have been accumulated preliminary reports demonstrating the potential effects of these PI drugs on COVID-19 patients.[86−88] 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 om this patient group. An early study by Zhu et al. reported the coinfection case of SARS-CoV-2 and HIV and suggested that "HIV patients should be regarded as vulnerable group".[89] However, this reported patient did not have previous antiviral therapy, which is less typical.[90] 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.[91] 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

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J. Proteome Res. 2020, 19, 4316−4326

Table 1

market status	potential candidate	target	IC_{50} (μM)	mechanism
natural product	baicalein (PDB code 6M2N)	Mpro	0.94[68] (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.[68]
approved	talampicillin	Mpro		Talampicillin and lurasidone showed a reliable binding pattern in Mpro and closed the active site of the enzyme.[70]
approved	lurasidone	Mpro		
approved	lopinavir	Mpro	12.01[92] (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[92] (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	nelfinavir	Mpro/ PLpro	0.77[75] (by cell-based assay)	The benzamide carbonyl group and octahydro-1*H*-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	valganciclovir	Mpro/ PLpro		Virtual binding shows that it can bind to Mpro and PLpro, so it may be a dual-enzyme inhibitor.[71]
approved	inarigivir	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[93] (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[93] (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	0.75uM[97] (by protein enzymatic assay)	The metastasis inhibitory factor of prostate cancer was found by chemical library screening, which confirmed that bromohexine 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[73] (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[66] (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 $\pm$ 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.[71] (virtual ligand screening)
approved	elsulfavirine	PLpro		Elsulfavirine 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]
approved	faldaprevir	PLpro		Faldaprevir is an investigational compound to treat chronic hepatitis C (HCV) and intercalate with the active site of PLpro, mainly by hydrogen-bond formation with residues Glu912 Asp909, Tyr1013, and Arg911.[71]
natural product	quercetin	Mpro		Quercetin is able to form complexes with the Mpro with good binding affinities by molecular dynamics (MD) simulations.[73]
approved	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.

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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 or the IV approach to inhibit TMPRSS2 because TMPRSS2 is critical for viral entry but with low toxicity. With the viral activation/entry mechanism by various extracellular proteases and the potential *in trans* regulation, the clinical use of chloroquine/hydroxychloroquine to inhibit cathepsin may prove to be futile,[22−24] as suggested by multiple experimental and clinical studies. Furthermore, chloroquine has also been proved lethal because of its dramatic cardiac toxicity. Bench-to-bed translation has never been more important and challenging at this moment of global outbreak of COVID-19. We look forward to witnessing more research efforts and collaborations for better understanding proteases and the resultant more effective antiviral treatments for COVID-19.

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Notes

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