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Host Cell Proteases Mediating SARS-CoV-2 Entry: An Overview

Mehdi Oubahmane¹, Ismail Hdoufane¹, Imane Bji², Nouhaila Ait Lahcen¹, Didier Villemin³, Rachid Daoud⁴, Achraf El Allali⁴ and Driss Cherqaoui^{1,*}

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Abstract: The outbreak of the SARS-CoV-2 virus in late 2019 and the spread of the COVID-19 pandemic have caused severe health and socioeconomic damage worldwide. Despite the significant research effort to develop vaccines, antiviral treatments, and repurposed therapeutics to effectively contain the catastrophe, there are no available effective vaccines or antiviral drugs that can limit the threat of the disease, so the infections continue to expand. To date, the search for effective treatment remains a global challenge. Therefore, it is imperative to develop therapeutic strategies to contain the spread of SARS-CoV-2. Like other coronaviruses, SARS-CoV-2 invades and infects human host cells *via* the attachment of its spike envelope glycoprotein to the human host cell receptor hACE2. Subsequently, several host cell proteases facilitate viral entry *via* proteolytic cleavage and activation of the S protein. These host cell proteases include type II transmembrane serine proteases (TTSPs), cysteine cathepsins B and L, furin, trypsin, and Factor Xa, among others. Given the critical role of the host cell proteases in coronavirus pathogenesis, their inhibition by small molecules has successfully targeted SARS-CoV-2 *in vitro*, suggesting that host cell proteases are attractive therapeutic targets for SARS-CoV-2 infection. In this review, we focus on the biochemical properties of host cell proteases that facilitate the entry of SARS-CoV-2, and we highlight therapeutic small molecule candidates that have been proposed through *in silico* research.

Keywords: COVID-19, SARS-CoV-2, Host cell proteases, TTSPs, Cathepsins, Furin, Small-molecule inhibitors, Proteolytic activation, and protease inhibition.

1. INTRODUCTION

Since the emergence of COVID-19 disease, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the entire world has had consequential health and socio-economic disruption. The World Health Organization (WHO) reported that infected individuals reached 422 million, with nearly 5.8 million deaths worldwide by 20th February 2022. SARS-CoV-2 is the seventh known Coronaviridae family member to infect humans [1] and the third documented member after the SARS-CoV (2003) and the Middle East respiratory syndrome coronavirus (MERS-CoV, 2014) that caused a pandemic and epidemic in the 21st century.

As with other coronaviruses, the genome of SARS-CoV-2 is approximately 30 kb in size and shares the same genomic characteristics, [5'-replicase (rep), spike (S), envelope (E), membrane (M), and nucleocapsid (N)-3'] [2]. The genome contains two long open reading frames (ORF), ORF1a

and ORF1b, covering more than two-thirds of the 5' end of the genome. These two ORFs are translated into two polyproteins, pp1a and pp1b and encode 16 nonstructural proteins (nsp1-nsp16) that form the viral replicase transcriptase complex (RTC). The 3' end of the genome contains the remaining ORFs, which span one-third of the genome and encode four viral structural proteins (S, M, E, and N) and the nine auxiliary proteins [3]. Among the structural proteins, the spike protein of SARS-CoV-2 is an essential viral protein that infects host cells using human angiotensin-converting enzyme 2 (hACE2) as a specific host receptor [4] and human proteases as activators [5].

Spike proteins coat the SARS-CoV-2 surface and the receptor-binding domain (RBD) of the Spike protein attached to hACE2, allowing the virus to enter the cell [6]. hACE2 is an ectoenzyme that regulates blood pressure and is highly expressed in the lungs, kidneys, heart, and gastrointestinal tract. Once attachment occurs, human proteases activate the S protein, facilitating viral entry into the cell. After entering the cell, the virus releases RNA, which is then translated to polyproteins and followed by viral RNA replication and

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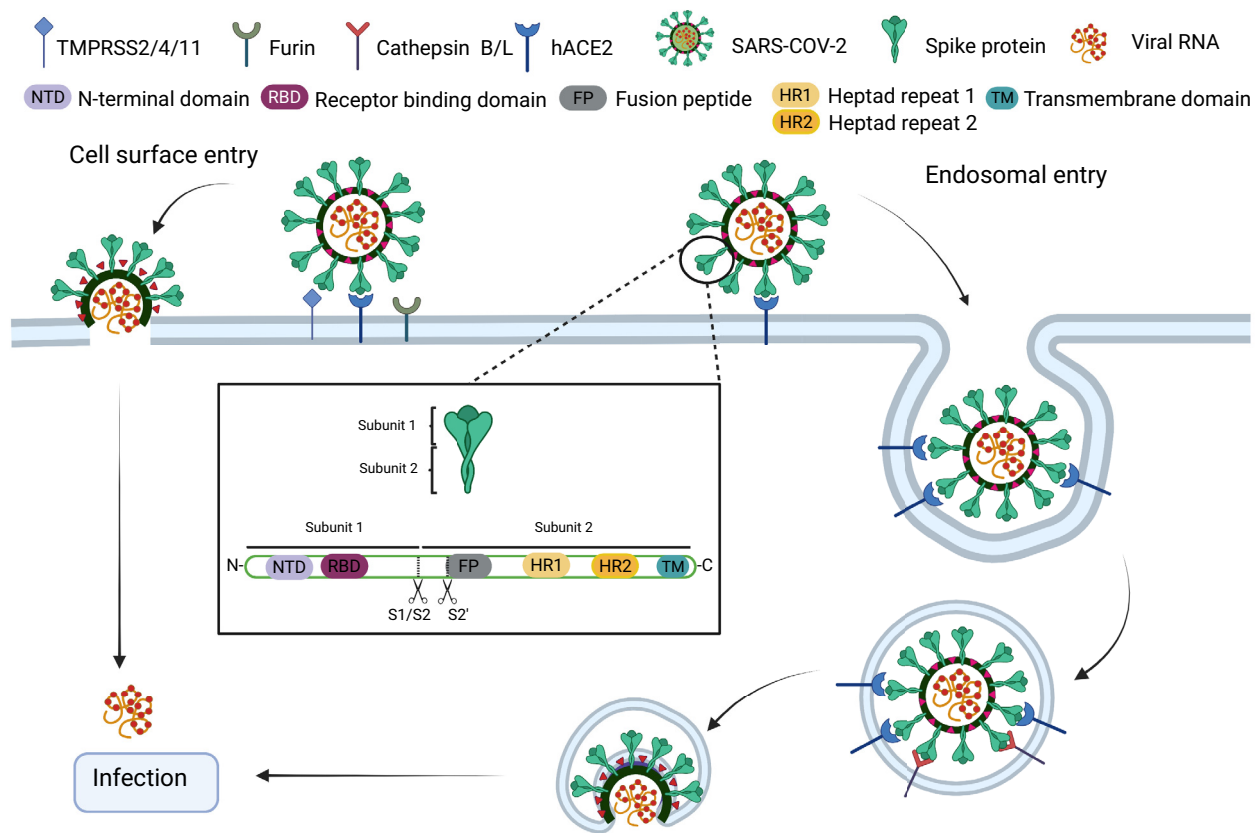


Fig. (1). Sketched view of the SARS-CoV-2 entry pathway, Fig. (1) was created with BioRender.com. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

transcription *via* cleavage of proteins and assembly of the RTC. The viral RNA is amplified in the host cell, and the structural proteins are generated, assembled, and packed before the viral particles are released [7].

The S protein of SARS-CoV-2 is the largest structural protein in the virus particles, with a total length of about 1300 amino acids. It is arranged in the form of homotrimers that protrude into the viral envelope, creating the corona shape of the SARS-CoV-2 virus [8]. The S protein is a type I transmembrane protein with a long ectodomain, a short transmembrane domain, and a short endodomain. A functional subunit (S1) is responsible for binding the host cell receptor, and a second domain (S2) is accountable for membrane fusion within the ectodomain. The S1 subunit consists of an N-terminal domain (NTD) and a receptor-binding domain (RBD) which binds to the hACE2 [9]. At the same time, S2 is a membrane-anchored subunit consisting of an N-terminal hydrophobic fusion peptide (FP), two heptad-repeat regions (HR1 and HR2), a transmembrane domain (TM), and a cytoplasmic tail [10, 11]. For S2 subunit fusion machinery activation, the S protein must be proteolytically activated by various host cell proteases capable of cleaving its ectodomain at specific sites. The S protein has two key cleavage sites: i) the S1/S2 site at the border between the two subunits, the dissociating S1 subunit and the S2 subunit undergoes a conformational change, and ii) the S2' site, lo-

cated within the S2 domain [12]. Host cell proteases are necessary for S protein cleavage, which can occur on the cell's surface before the viral entrance, inside endosomes during the viral entrance, and in the cytoplasm during viral protein production by the infected cell [8]. SARS-CoV-2 can enter host cells *via* two pathways based on the availability of proteases essential for activating the Spike protein. The first pathway occurs when no spike activating proteases are expressed on the host cell surface; then, SARS-CoV-2 can enter the cell in an endocytic way and can be cleaved and activated by pH-dependent proteases. While the second pathway is spike cleaved and activated when a spike activating protease is coexpressed with hACE2 on the host cell surface (Fig. 1) [13, 14]. Several host proteases have been found to start the S protein *in vitro* at either cleavage sites and play a pivotal role in the rapid spread and severe symptoms of SARS-CoV-2 [5]. Therefore, blocking proteases implicated in the proteolytic activation of SARS-CoV-2 S has been highlighted as potential intervention targets.

Given what is known about the human proteases that facilitate viral entry, several reports have studied and proposed small molecule drugs to inhibit and decrease their enzymatic activity to limit SARS-CoV-2 infection. In this review, we explore these proteases and gather the therapeutic small-molecule candidates proposed through *in silico* research.

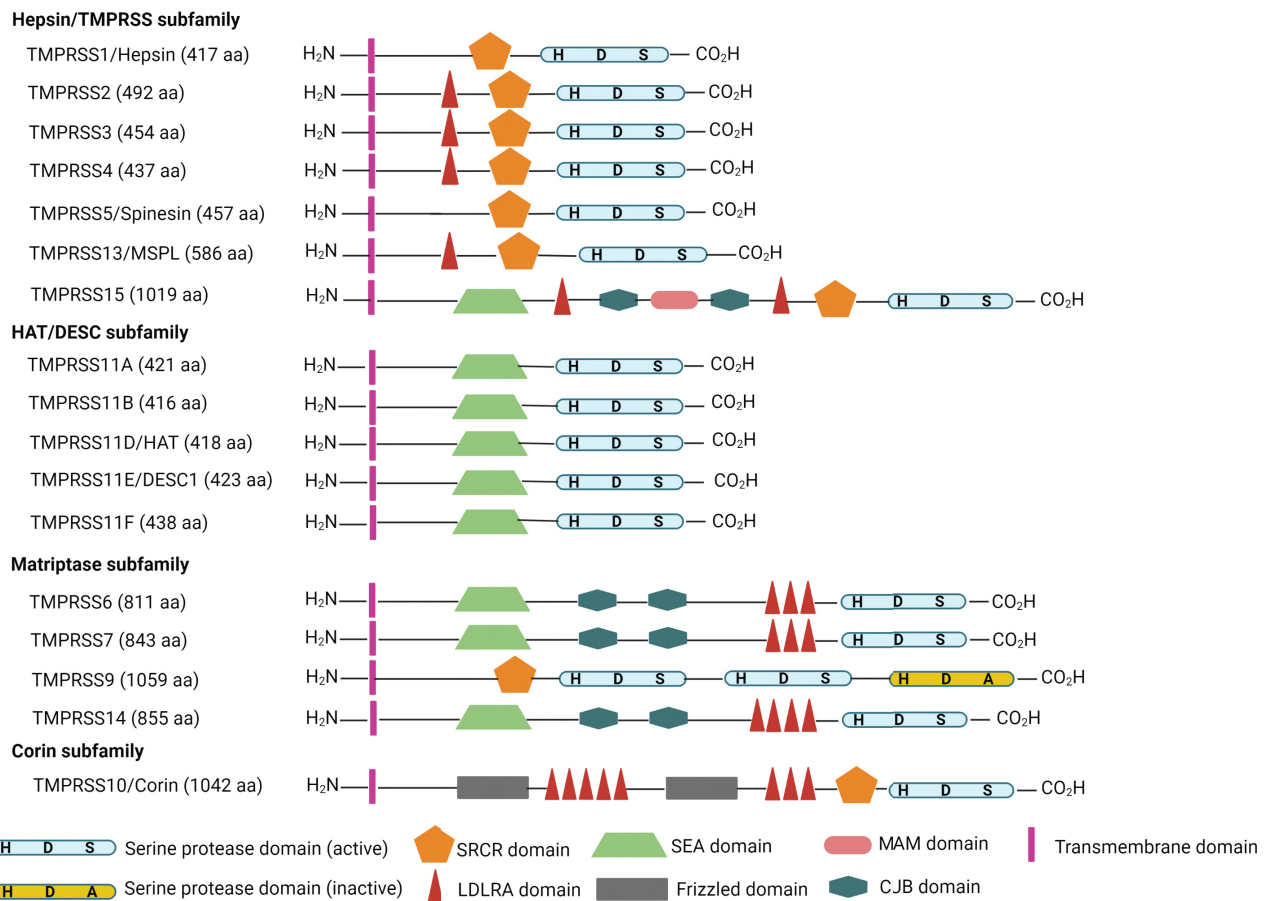


Fig. (2). Schematic representation of the human type II transmembrane serine proteases (TTSPs) structures. aa: amino acids; CUB: Complement C1s/Clr, urchin, embryonic growth factor, and bone morphogenetic protein-1; DESC1: Differentially expressed in squamous cell carcinoma; HAT: Human airway trypsin-like protease; LDLRA: Low-density lipoprotein receptor A; SEA: Sea urchin, sperm protein, enteropeptidase, and agrin; SRCR: Scavenger receptor, cysteine-rich; MAM: Meprin, A5 protein, and receptor protein tyrosine phosphatase μ ; MSPL: Mosaic serine protease large form; TMPRSS: Transmembrane serine protease. The amino-terminal and carboxyl-terminal are indicated by NH₂ and CO₂H, respectively. Fig. (2) was created with BioRender.com. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

2. TYPE II TRANSMEMBRANE SERINE PROTEASES (TTSPS)

Many host cell proteases moderate SARS-CoV-2 entry among the candidates for the direct fusion of the virus at the plasma membrane surface: type II transmembrane serine proteases (TTSPs) [15]. The family of serine proteases is expressed in multiple organs and tissues throughout the body. They involve various biological processes such as epithelial development, cellular iron homeostasis, blood control, and immune responses. Remarkably, some TTSPs expressed in the human airways are known to facilitate the entry and spread of not only SARS-CoV-2 but also other human coronaviruses like the SARS-COV and the MERS-COV viruses [16]. The TTSPs family has 17 proteases subdivided into four different subfamilies: (i) the hepsin/TMPRSS subfamily, (ii) the HAT/DESC subfamily, (iii) the Matriptase subfamily, and (vi) the Corin subfamily (Fig. 2) [17]. These proteases share a common structural architecture: a cytoplasmic N-terminal region, a transmembrane region, and an extracellular proteolytic region [18-20]. The extracellular part has a low-density lipoprotein receptor

class A (LDLRA), a scavenger receptor cysteine-rich (SRCA) domain, and a serine protease domain [21]. The proteolytic cleavage mechanism of TTSPs is highly conserved. Its activity depends on a catalytic triad constituted of the three amino acids histidine, aspartic acid, and serine. The catalytic mechanism proceeds in two steps, acylation/deacylation, where the acylation step is implicated in forming a covalently linked enzyme-peptide intermediate and losing a peptide fragment. Then a deacylation step is marked by water's nucleophilic attack on the intermediate, causing the peptide's hydrolysis [14, 22]. At the same time, the substrate-binding pocket determines the enzymatic specificity of each TTSP. TTSPs are reported to be single-chain, inactive zymogens that require activation by cleaving a positively charged residue (Arginine or Lysine) in the conserved activation motif between the pro-domain and catalytic domain to release the active mature protein. Upon activation, they retain membrane attachment *via* disulfide linkages between the catalytic and transmembrane domains. TTSPs include TMPRSS1, TMPRSS2, TMPRSS3, TMPRSS4, TMPRSS6, TMPRSS13 [23], and TMPRSS14 and are capable of auto-activation, indicating that their respective zy-

mogens have a baseline activity [24]. Therefore, there are only five available 3D structures (TMPRSS11E (2OQ5), TMPRSS15 (4DGJ), TMPRSS13 (6KD5), TMPRSS1 (5CE1) and TMPRSS2 (7MEQ)) among all TTSPs in the Protein Data Bank (PDB) database. According to reports, TMPRSS2 is the one that has been the host factor of ACE2 that promotes the proteolytic activation and spread of SARS-CoV-2 [25]. However, few articles reported that other TTSPs mediate the SARS-CoV-2 entry, such as TMPRSS4, TMPRSS13, TMPRSS11D, TMPRSS11E, and TMPRSS11F. This section will concentrate on three TTSPs (TMPRSS2, 4, 11D) that have been considerably studied in SARS-CoV-2 to date.

2.1. TMPRSS2

TMPRSS2 has been recently spotlighted as a favorable antiviral drug target for SARS-CoV-2 because of its highly permissive function in the entry and spread of SARS-CoV-2. TMPRSS2 is notably localized in the human epithelial tissues of the respiratory, genito-urinary, and gastrointestinal tract systems. It is coexpressed with the virus receptor hACE2 in lung tissues [26-28]. TMPRSS2 mediates viral entry *via* two distinct mechanisms; the proteolytic cleavage of ACE2, which enhances viral uptake [16], and the spike protein cleavage, which activates the glycoprotein for host cell entrance [29, 30]. TMPRSS2 activates the S protein of SARS-CoV-2 at two possible cleavage sites: Arg689/Ser690 at the S1/S2 site and Arg815/Ser816 at the S2' site, resulting in two different fragments of the S protein [31] and reducing the amount of activity of TMPRSS2. Thus, the rate of virus implantation, local replication, dissemination, and secondary replication is considerably decreased. TMPRSS2 knockout mice show no phenotype in the absence of infection, indicating that suppressing TMPRSS2 may not be linked with significant undesired side effects [32]. Thus, inhibiting TMPRSS2 function using clinically proven small-molecule TMPRSS2 inhibitors will contribute to COVID-19 therapy.

Some studies have found that males had considerably greater death and hospitalization rates than females because of COVID-19. The expression of TMPRSS2 is increased by high doses of estrogen. As a result, blocking androgen receptor activity or stimulating estrogen pathways can prevent SARS-CoV-2 entry. Several FDA-approved antiandrogens such as darolutamide, apalutamide, and enzalutamide have been proposed to attenuate SARS-CoV-2 infection [33-38].

Several clinical trials are ongoing to test serine protease inhibitors. Nafamostat mesylate is a potent matriptase inhibitor and an approved anticoagulant in Asian countries [39, 40]. Camostat mesylate was licensed in Japan for chronic pancreatitis treatment and postoperative reflux esophagitis. Camostat is rapidly processed to 4-(4-guanidinobenzoyloxy) phenylacetic acid (GBPA), which inhibits TMPRSS2 and SARS-CoV-2 entry, but somewhat less efficiently than camostat [41-44]. Gabexate is an investigational drug used to prevent blood clots and lower the generation of inflammatory cytokines. AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) is a cholesterol regulator. Bromhexine is used for respiratory disorders characterized by viscid

or excessive mucus [45]. At the same time, aprotinin is a trypsin inhibitor utilized as an anti-inflammatory and anti-coagulant. Additional work has discovered a reversible, small-molecule ketobenzothiazole (MM3122) that has improved activity over nafamostat and camostat [46].

Most of the computational studies were published before the characterization of the TMPRSS2 structure. They used homology modeling to model a 3D structure, did a virtual screening of different datasets (FDA-approved drugs, ZINC, and natural compounds), molecular docking, and molecular dynamic simulation approaches to target the TMPRSS2 enzyme, and reported the suitability of several small molecules as candidate drugs (Table 1). Further computational studies have revealed that camostat, nafamostat, and gabexate block TMPRSS2 by creating an irreversible covalent connection with Serine441 at the catalytic site. Compared to other guanidinobenzoyl-containing drugs, nafamostat creates a better binding with TMPRSS2 because it can clamp two positively charged groups (guanidinium group and amidinium group) in either a forward or reverse manner, with the forward setting being more stable [47]. Contrarily, some reports indicated the unlikely binding of bromhexine to the catalytic site and proposed that bromhexine might bind into a putative allosteric binding pocket [48].

Because of the inherent problems connected with solving the crystal structures of membrane-bound proteins, there is no highly selective inhibitor for TMPRSS2 or any other TMPRSS subfamily members. However, the lack of selectivity poses a challenge in developing a safe and selective inhibitor that targets TMPRSS2 and avoids the total inhibition of serine proteases (Table 1).

2.2. TMPRSS4

TMPRSS4, also known as cyclase-associated actin cytoskeleton regulatory protein 2 (CAP2), is a protein of 437 amino acid residues. Its overexpression has been linked to cell proliferation and metastasis in various malignancies. TMPRSS4 with TMPRSS2 was involved in SARS-CoV-2 entry in human small intestine enterocytes [72]. At present, no X-ray crystallographic structure defined TMPRSS4 has been published, and no medicines are currently available to treat this protein. Only two inhibitors have been described in the literature: a 2-hydroxydiarylamide derivative with an IC_{50} of 6 μ M [73, 74] and Tyrosylleutide (TSL) with an IC_{50} of 20 μ M [75]. Nonetheless, their pharmacological effects, toxicity, and safety are unclear. Furthermore, only one *in silico* report studied and identified four TSL derivatives that may have stronger inhibitory action than the TSL molecule [76].

2.3. TMPRSS11D

TMPRSS11D, also called human airway trypsin-like protease (HAT), is coexpressed with ACE2 in bronchial epithelial cells and pneumocystis [77, 78]. It is well known that TMPRSS11D was highly produced in human cervical cancer cells. However, the expression, biological role, and activation mechanism of TMPRSS11D have not been thor-

Table 1. Small molecules as candidate drugs targeting TMPRSS2.

Used Techniques	Outcomes	References
Homology modeling QM/MM-MD	Structural analysis of TMPRSS2 bonding with Camostat and its metabolite GBPA	[49]
Homology modeling QM/MM-MD	Structural analysis of TMPRSS2 bonding with Nafamostat +Camostat and its metabolite GBPA	[47]
Homology modeling + Pharmacophore + Docking + MD	Four non-covalent inhibitors (Otamixaban, UK-1, NCGC00386945, and NCGC00487181) against TMPRSS2 are still less effective than camostat and nafamostat	[50]
Homology Modeling + MD	Non-target binding study of nafamostat and camostat	[51]
MD	MD analysis of binding nafamostat, camostat, gabexate, Bromhexine	[52]
Homology modeling + Virtual screening + Docking	156 small molecules proposed to have an excellent inhibition profile against TMPRSS2	[53]
Homology modeling + Docking + MD	Allosteric binding site BH	[48]
Homology modeling + Docking + MD	Three phytochemicals (Niazirin, Quercetin, and Moringyne) are better than camostat	[54]
Homology modeling + Docking + MD	Cepharanthine is better than camostat	[55]
Homology modeling + Pharmacophore+ Docking + MD	ZINC64606047 and ZINC05296775 are better than nafamostat	[56]
Docking + Biological Assay	Hesperidin and hesperetin	[57]
Homology modeling + Virtual Screening + Docking + MD	6 FDA drugs (Valrubicin, lopinavir, fleroxacin, alvimopan, arbekacin, dequalinium) against TMPRSS2 and ACE2	[58]
Homology modeling + Virtual Screening + Docking + MD	camostat (ZINC203686879) and Differin (ZINC3784182) have a good inhibition profile	[59]
Homology modeling + Virtual Scree- ning+Docking + MD	Talampicillin, Lurasidone, Rubitecan, and Loprazolam may have a potential inhibition against TMPRSS2 and Mpro	[60]
Homology modeling + Virtual Screening + Docking + MD	Three Fluorine based quinolines 100955749, 101186842, and 102430872 may have a potential inhibition against TMPRSS2	[61]
Homology modeling + Docking + MD	bisdemethoxycurcumin (BDMC), carvacrol, and thymol have better inhibition than camostat	[62]
Homology modeling + Docking + MD	Robinin, Daidzin, and Hydroxytuberosone have potential inhibition profile against TMPRSS2	[63]
Homology modeling + Docking + MD	structural analysis of TMPRSS bonding with camo + nafamostat+ bromhexine	[64]
Homology modeling + Docking + MD	Structural analysis of TMPRSS2 binding with nafa+camostat+gabexate +sivelestat	[65]
Homology modeling + Docking + MD	5 phytochemicals against TMPRSS2	[66]
Homology modeling + Docking + MD	Withaferin A + Withanolide D has a dual inhibition profile targeting TMPRSS2 and ACE2	[67]
Docking + MD	Ibrutinib and zanubrutinib may have multiple inhibition profiles against TMPRSS and viral proteins	[68]
Homology modeling + Docking + MD	Withanone and Withaferin A may have a better inhibition profile than camostat	[69]
Homology modeling + Docking + MD	benzquercin proposed as a robust TMPRSS2 inhibitor	[70]
Homology modeling + Docking + MD	Leupeptin may be a good alternative for camostat	[71]

oughly investigated [79]. The S protein cleaves differently by TMPRSS11D than by TMPRSS2 [80]. Therefore, the 3D structure of the protein and its selective inhibitor has not yet been identified. A 4-amidinobenzylamide moiety incorporated with norvaline has shown a strong inhibition with an inhibition constant of 15nM and higher selectivity to HAT over the coagulation proteases thrombin and factor Xa or fibrinolytic plasmin [81].

3. CYSTEINE CATHEPSINS B AND L

Cathepsins form the most abundant lysosomal membrane protein family that activates in acidic environments (pH 4.5 to 5.5) [82, 83]. The 15 known cathepsins are classified into three subfamilies based on the amino acids of their catalytic sites (i) Serine cathepsins (A and G), (ii) aspartic cathepsins (D and E), and (iii) cysteine cathepsins (B, C, F,

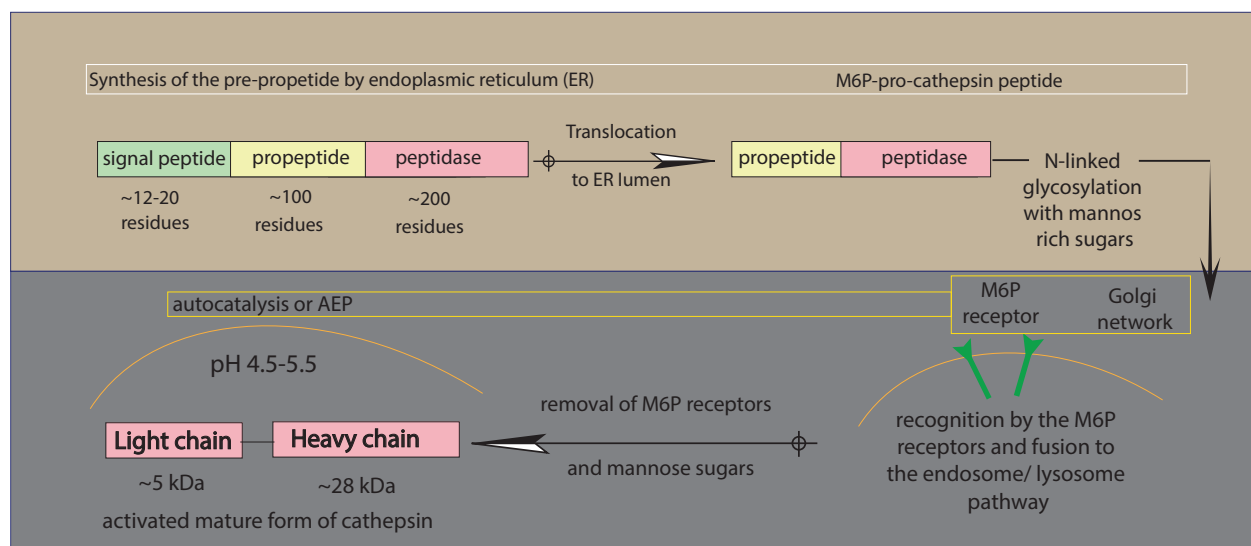


Fig. (3). The transport pathway of cathepsins explains the synthesis of the pre-propeptide by the endoplasmic reticulum (ER), followed by translocation to the ER lumen where the M6P-procathepsin peptide is formed. M6P receptors recognize this form, and proteolytic processing of late endosomes yields activated mature cathepsins. (*A higher resolution / colour version of this figure is available in the electronic copy of the article.*)

H, K, L, O, S, V, W, and X) [84]. Among all cathepsins, an abundance of cathepsin B and L is noticed [85]. Their stimulation at low pH explains their crucial role in the endosome/lysosome pathways, where they are indeed present in high concentrations [86]. This further emphasizes their involvement in mediating many cellular events, including physiological and pathological processes, and thus, their association with numerous diseases, including COVID-19 [87]. Recent studies have suggested the inhibition of cathepsins L and B as an efficient strategy against coronavirus disease given their importance in the entry and priming of SARS-CoV-2, particularly the proteolysis of the coronavirus surface spike protein [88, 89].

Cathepsins B and L attain the lysosome *via* the endosome/lysosome pathway, where the mature form is achieved through a step-by-step process [90]. First, the pre-propeptide (about 20 amino acids N-terminal signal peptide) is synthesized by the endoplasmic reticulum (ER) and transported in a co-translation manner to the ER lumen, where it is processed. As a result, pro-cathepsins are generated and are subjected to N-linked glycosylation with mannose-rich sugars forming M6P-pro-cathepsin peptides [91]. This is later delivered to the Golgi apparatus, which is recognized by the M6P receptors and translocated to the late endosome. Afterward, the lysosomal acid phosphatase in endosomes/lysosomes liberates the M6P receptors and the phosphate group from mannose sugars. Eventually, autocatalysis in the lysosomal acidic environment or proteolysis through cathepsin D or asparagine endopeptidase (AEP) known as legumain is required to remove the N-terminal propeptide and to generate the active mature forms of cathepsin B and L in the lysosome. The structure of cathepsins at this phase consists of two chains that remain attached to each other by disulfide bridges: a heavy chain (~28 kDa) and a light chain (~5 kDa) (Fig. 3) [92].

In cathepsins B and L, cysteine amino acid and histidine residue form a dyad in the catalytic site where the hydrolysis of peptide bonds occurs. The sulfhydryl on the cysteine side chain acts as a nucleophile. However, the histidine residue provides a proton, which increases the nucleophilicity of the cysteine making cysteine cathepsins high nucleophile proteins [93]. The catalytic dyad's ionic form -thiolate/imidazolium- is activated in an acidic environment. An asparagine amino acid residue secures the correct orientation of imidazolium through a hydrogen bond [94]. The thiolate group of the cysteine residue performs the nucleophilic attack of the carbonyl group of the peptide bond, releasing the amine or amino terminus portion of the substrate throughout generating a hydrogen bond stabilized tetrahedral intermediate. The eventual contribution of the water molecule hydrolyses the thioester bond, releasing the carboxylic acid moiety from the remaining substrate portion and healing of the cathepsin [95].

The dual localization of cathepsins at both intra- and extracellular levels is closely related to their emergence in biological and pathological processes [96]. In the case of abnormal expression, cathepsins can be regulated by endogenous inhibitors, the difference in temperature, physiological pH, oxidation, or small molecule inhibitors that can be synthetic or of natural origin. To date, enormous efforts have been made to treat diseases involving cathepsins. For instance, antimicrobial peptides have shown great results in inhibiting cathepsins, including cathepsin B and L [97]. Flavonoids and other chemical families such as organosulfur compounds and proteoglycans also proved competitive inhibition of cathepsins [98]. However, it remains to be seen if the challenges related to the broad-spectrum inhibition of these classes of therapeutic molecules can be overcome. In this review, we found it helpful to emphasize that many compounds are subjected to numerous *in silico* predictions

(Table 2). However, more extensive studies must be conducted to discover effective cathepsin inhibitors.

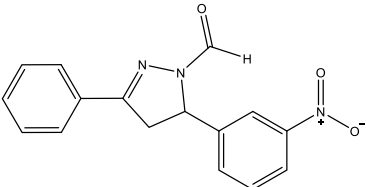
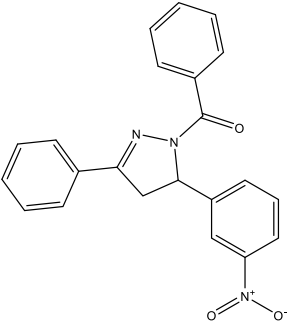
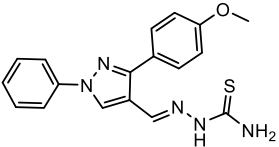
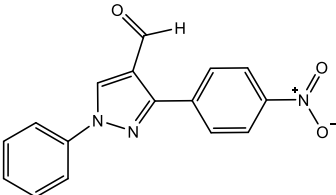
4. FURIN AND ITS INHIBITORS

Furin is a serine protease that belongs to the proprotein convertase subtilisin kexin (PCSK) family [108, 109]. This protease is expressed everywhere in organs and tissues, such as the gastrointestinal tract, lung, central nervous system, and reproductive tissues [109]. Furin plays a prominent role in viral pathogenesis in all cells and efficiently cleaves polybasic or multibasic sites identified in the influenza virus (*e.g.*, influenza virus subtypes H5 and H7). It is more reactive than other proprotein convertases involved in viral activation, such as PC-4, PC-5, and PC-7 [110]. Mechanistically, after viral attachment, the furin site is implicated through polymerase slippage during replication. Following the release of the SARS-CoV-2 genome, many researchers pinpointed a four-amino acid insert beginning with proline (SPRRAR|S) at the junction of the spike protein's receptor-binding (S1) and fusion (S2) domains [109, 111, 112]. The R-X-X-R motif is a possible cleavage target and a classical

binding site for the protease furin [112]. The insert is a polybasic site thought to be part of the continuing pandemic's proximal genesis.

In addition to the classical Furin cleavage site (R-X-X-R), Yue Zhang *et al.* predicted that Furin's position is not fixed [113], and the enzyme has been observed to be extracellularly secreted [113]. Furin's location activates the S protein during viral morphogenesis, as observed in the instance of MERS-CoV, which has the R-X-X-R motif in the S1-S2 cleavage site. This location is not seen in SARS-CoV, implying that the S protein activation at the S2' site by enzymes such as TMPRSS2 or furin-like enzymes before viral entrance into the new cell is required [114]. A Furin-like recognition site has recently been discovered in the S1/S2 region of SARS-CoV-2. As an outcome, S protein activation within the host cell before virus egress may occur, eliminating the potential for virus activation before attacking a new host cell [110]. Regarding the significance of furin and other PCSK enzymes in viral pathogenesis, furin inhibitors can be explored to prevent viral spread and reduce SARS-CoV-2 infectivity.

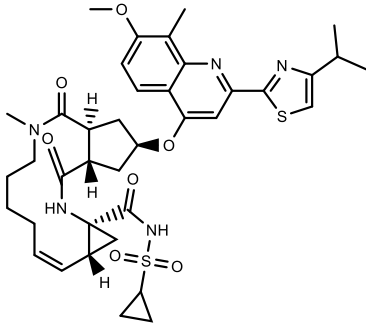
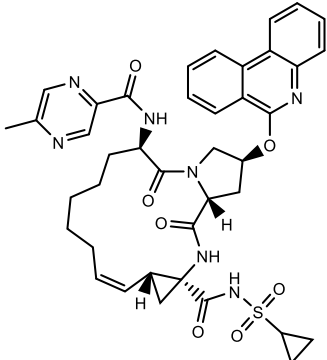
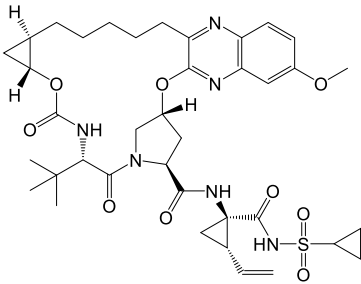
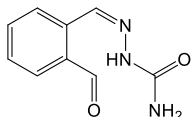
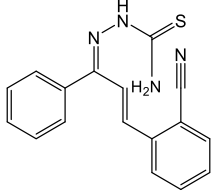
Table 2. Compounds with *in silico* have proven activity against cathepsins.

Inhibitor	2D Structure	Target	<i>In silico</i> Method	References
Pyrazole Derivatives				
N-formyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline		- Cathepsin B - Cathepsin H	- SAR studies - Docking studies	[99]
N-benzoyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline		- Cathepsin B - Cathepsin H	- SAR studies - Docking studies	[99]
1-((3-(4-Methoxyphenyl)-1-phenyl-1H-pyrazol-4-yl)methylene)thiosemicarbazide		- Cathepsin B - Cathepsin H	- SAR studies - Docking studies	[100]
3-(4-nitrophenyl)-1-phenyl-1H-pyrazole-4-carbaldehyde		- Cathepsin L	- SAR studies - Docking studies	[100]

(Table 2) contd...

Inhibitor	2D Structure	Target	In silico Method	References
Nitrile-based Inhibitors				
(2S)-N-[(1R,2R)-2-(benzyloxy)-1-cyanopropyl]-2-([1,1'-biphenyl]-4-yl)formamido-3-(3-chlorophenyl)propanamide		- Cathepsin B	- Molecular Dynamics Simulations - SAR studies	[101]
N-((S)-1-(((1R,2R)-2-(benzyloxy)-1-cyanopropyl)amino)-3-(3-chlorophenyl)-1-oxopropan-2-yl)-3-(pyridin-3-yl)benzamide		- Cathepsin L - Cathepsin H	- Molecular Dynamics Simulations - SAR studies	[101]
Curcumin Derivatives				
(1E,4Z,6E)-5-hydroxy-1,7-bis(4-methoxyphenyl)hepta-1,4,6-trien-3-one		- Cathepsin L - Cathepsin B	- Bioactivity and ADMET generation tools - Molecular docking	[102]
Food Protein				
Lactoferrin		- Cathepsin L	- Molecular docking	[103]
Aurone-1,2,3-triazole Hybrids				
2-([(2E)-5-methyloxolan-2-ylidene]methyl)-6-[(1-phenyl-1H-1,2,3-triazol-4-yl)methoxy]-2,3-dihydro-1-benzofuran-3-one		- Cathepsin B	- Molecular docking	[104]
Quinazoline Derivatives				
(E)-8-(4-fluorobenzylidene)-4-(4-fluorophenyl)-2-phenyl-5,6,7,8-tetrahydroquinazoline		- Cathepsin L - Cathepsin B	- Molecular docking	[105]

(Table 2) contd...

Inhibitor	2D Structure	Target	<i>In silico</i> Method	References
FDA-approved Antiviral Drugs				
Simeprevir				
Paritaprevir		Dual inhibition against the main viral protease of SARS-CoV-2 and the host calpains/cathepsins	- homology modelling - virtual screening	[106]
Grazoprevir				
Semicarbazones and Thiosemicarbazones Derivatives				
[(Z)-[(2-formylphenyl)methylidene]amino]urea		- Cathepsin L - Cathepsin B - Cathepsin H	- QSAR studies - Molecular docking - ADMET prediction	[107]
[(E)-[(2E)-3-(2-cyanophenyl)-1-phenylprop-2-en-1-ylidene]amino]thiourea		- Cathepsin L - Cathepsin B - Cathepsin H	- QSAR studies - Molecular docking - ADMET prediction	[107]

4.1. *In silico*-based Studies Targeting Furin

Furin inhibitors have been considered a potential treatment platform against SARS-CoV-2 infection. Although further study is needed to prove this assumption experimentally, inhibition of furin or furin-like enzymes may provide a viable antiviral platform. Indeed, it has recently been proven

that host cells infected with various viruses activate an interferon-based activity to inhibit the enzymatic activity of furin-like enzymes [115]. It was also shown that virus infection causes the overexpression of certain receptors that inhibit furin trafficking in post-Golgi compartments [116]. Multiple peptide-based and small-molecule inhibitors targeting furin/Pc have recently been designed to act as putative

Table 3. A list of Furin protease inhibitors against COVID-19.

Inhibitor	Mechanism of Action	<i>In Silico</i> Method	References
Mozenavir (DMP-450)	Furin with mozenavir had the lowest binding energy of -12.05 kcal/mol	Molecular Docking	[124]
Theta-defensins (TDs)	The low electrostatic potential of furin active cleft and high electrostatic potential of the TDs show that TDs can function as furin inhibitors.	Sequence Comparison and Molecular docking	[125]
Limonin, gedunin, eribulin, pedunculagin, limonin glycoside, and betulinic acid	Potential phytochemicals inhibiting the proteolytic cleavage activity of furin and TMPRSS2.	<i>In-silico</i> molecular docking screening	[126]
Heparin	Heparin may exercise its antiviral activity by masking the S1/S2 site. This prevents cleavage of furin and inhibits the activation of the postfusion conformation of the spike glycoprotein.	Molecular dynamics (MD) simulation	[127]

antiviral agents. Several of these inhibitors can hinder the maturation of viral envelope proteins and, therefore, their fusion activity in different viruses. [110]. Furthermore, it has previously been discussed that furin-interfering medications include 1-antitrypsin Portland (1-PDX) [117, 118], crude drugs which contain honokiol [119], chloromethylketone (CMK), and naphthofluorescein demonstrated antiviral effects on SARS-CoV-2-infected cells by reducing virus replication as well as cytopathic effects [120], Decanoyl and obatoclox inhibitors [121, 122], and peptides and peptidomimetics [123]. To the best of our knowledge, no furin inhibitor small molecules with high specificity and good effects have been discovered. Therapeutic inhibition of furin may be used as a therapeutic approach for COVID-19. A list of furin protease inhibitors is listed in Table 3.

5. TRYPSIN (TRYPTASE)

Trypsin is a serine endopeptidase that functions as a digestive enzyme. It is expressed primarily in the digestive and respiratory cells [128]. Although trypsin is not very selective for cleavage sites on the S protein, it primarily recognizes and cleaves within the amino acid chain of the proteolytic sensitive activation loop at the boundary of S1/S2 located in the spike protein [129]. Although the proteolytic activation mechanism at the S1/S2 interface and S2 site is unknown, the multibasic S1/S2 site is required for SARS-CoV-2 virus infection in lung cells [130]. Phylogenetic analysis indicated that cleavage of the SARS-CoV-2 S protein at the S1/S2 location might be related to priming by trypsin-like protease activity [131]. Trypsin and TMPRSS2 are both essential for activating syncytium formation during SARS-CoV-2 S protein expression. Thus, the S1/S2 location may be tailored to change viral pathogenicity and invasion [130]. Compared to other coronavirus subtypes, the cleavage potential of trypsin was more efficient for SARS-CoV-2 than for different coronavirus subtypes [25, 131]. As an outcome, trypsin also contributes to the cleavage of the S-glycoprotein. It may play a role in both the early viral entry phase and the formation of the late inflammatory cascade, suggesting a possible therapeutic target of SARS-CoV-2.

Several natural and synthetic inhibitors can either reversibly or irreversibly inactivate trypsin. Certain trypsin inhibitors may act as favorable prophylactic agents to prevent COVID-19 manifestation and progression by intercepting the entry of SARS-CoV-2 and preventing subsequent inflammation, coagulopathy, and severe dysfunction of multiple organs in vulnerable individuals. Recently, trypsin inhibitors have been proposed as prospective antiviral medications for treating COVID -19. Table 4 provides a list of trypsin protease inhibitors.

6. FACTOR XA

The coagulation protein factor Xa is a serine protease produced from the hydrolysis of its precursor factor X *via* two major routes [138, 139]. The extrinsic pathway occurs at the surface of injured endothelium and macrophages and in which factor VII /VIIa activates factor X in combination with a membrane-bound cofactor, tissue factor. The other intrinsic process involves a membrane-bound tenase complex composed of factor IXa, its cofactor factor VIIIa, and calcium ions activating factor X on the platelet surface. Factor Xa then triggers prothrombin to thrombin, resulting in blood coagulation [140]. A Factor X homolog was revealed to cleave the viral S protein at the S1/S2 boundary, allowing the virus to enter host cells [141]. The effect of factor X in the pathogenesis of SARS-CoV-2 has not been investigated in any study to date.

7. CLINICAL TRIALS OF DRUG CANDIDATES THAT TARGET HOST CELL PROTEASES

In order to face the pandemic, several strategies could help in the discovery of potent SARS-CoV-2 inhibitors. One of the promising strategies is drug repurposing (or drug repositioning) which seeks to find new uses for an approved or experimental drug unrelated to its original indication. The main advantages of this strategy are that it is less time-consuming, less expensive, and has a lower risk of failure because drug safety has been tested. Therefore, this strategy can bypass the pharmacokinetic, pharmacodynamic, and

Table 4. A list of trypsin protease inhibitors against COVID-19.

Inhibitor	<i>In silico</i> Method	Outcomes	References
<i>Cassia obtusifolia</i> trypsin inhibitor (COTI)	Molecular Docking	Formation of a complex between trypsin and COTI. High structural affinities between the inhibitor and the enzyme.	[132]
Trypsin inhibitors from tamarind seed (pTTI)	Molecular Dynamic simulations	Best Interaction Energy (-301.0128 kcal.mol ⁻¹). Strong interactions between pTTI and trypsin, which are the highest, are shown by the residues Ile (54), Pro (57), Arg (59), Arg (63), and Glu (78) of pTTI.	[133]
Curcumin	Molecular Docking and <i>in vitro</i>	Curcumin bonded into the pocket of trypsin and occupied the active site. The curcumin molecule forms mild interactions with amino acid residues in the active site causing its conformation to change from linear to U-shaped.	[134]
2-propanol	Molecular Docking and Molecular Dynamics	Hydrophobic interactions and hydrogen bonds govern trypsin-2-propanol bonds. The lowest binding free energy of the trypsin-2-propanol complex was found to be -3.69 kcal. mol ⁻¹ .	[135]
Ethylene glycol (EG)	Molecular Docking and Molecular Dynamics	Molecular docking revealed that EG interacts with amino acid residues of trypsin <i>via</i> hydrogen bonding and van der Waals interactions. Ethylene glycol was bonded to trypsin amino acids by hydrogen bonding with Asn 25, Gly 23, Thr 26, Val 27 and van der Waals forces with Arg 117. Molecular dynamics results showed that ethylene glycol functions as a trypsin stabilizer and activator.	[136]
Hesperetin (HES), Luteolin (LUT), Quercetin (QUE), Catechin (CAT), Rutin (RUT)	Molecular Docking and Molecular Dynamics	Hydrophobic interactions (π -stacking hydrophobic) between trypsin and flavonoids were predominant besides hydrogen binding, which is also involved in forming the flavonoid-trypsin complex. LUT was the best trypsin inhibitor (<i>e.g.</i> , lower IC ₅₀ , 45.20 \pm 1.00 μ M).	[137]

Table 5. List of drugs identified at <https://clinicaltrials.gov/> related to the host cell proteases for COVID-19 treatment.

Drug Name	Original Mechanism	Clinical Trials ID (Status)	Clinical Condition	Beginning / Estimated End	Phase
Camostat	Serine protease inhibitor	NCT04647497 (completed)	155 participants, 600mg*4 per day for 14 days	9-11-2020/26-03-2021	3
Nafamostat	Serine protease inhibitor	NCT04390594 (completed)	186 participants, 0,1-02mg/Kg/h	13-08-2020/12-08-2021	3
Bromhexine	Antioxidant	NCT04405999 (completed)	50 participants	14-05-2020/31-08-2020	4
Enzalutamide	Androgen receptor antagonist	NCT04456049 (not yet recruiting)	90 participants, 40mg daily for 28 days	-	2
Atovaquone	Antimalarial agent	NCT04456153 (completed)	60 participants, 1500mg twice per day for up to 10 days	22-07-2020/31-01-2021	2
Hydroxychloroquine	Anti-malarial	NCT04466540 (completed)	1372 participants, 400mg twice on the first day, followed by 400mg up to 7 days	12-05-2020/28-09-2021	4
Chloroquine	Anti-malarial	NCT04303507 (completed)	4646 participants, 10mg base/Kg followed by 155 mg daily for 3 months	29-04-2020/22-03-2022	Not applicable
Proxalutamide	Androgen receptor antagonist	NCT04728802 (completed)	645 participants, 300mg per day	01-02-2021/ 15-04-2021	3
Canrenoate potassium	Aldosterone antagonist	NCT04912011 (recruiting)	50 participants, 200 mg dissolved in 100 ml of 0.9% sodium chloride intravenously twice a day for 7 days.	01-01-2021/31-08-2021	4
Clofazimine	Antileprotic	NCT04465695 (recruiting)	81 participants	14-07-2020/30-06-2021	2
Arbidol	Antiviral agent	NCT04260594 (completed)	236 participants, 2 tablets three times per day for 14-20 days	08-02-2020/12-03-2020	4
Prolastin	Alpha-1 proteinase inhibitor	NCT04495101 (completed)	100 participants, 120mg/Kg up to 29 days	29-07-2020/26-04-2021	2

toxicity studies, leading to direct entry into phase II or III clinical trials and accelerating the drug development process. Table 5 presents approved drugs or clinical candidates that inhibit host cell proteases in COVID-19.

CONCLUSION

Since the COVID -19 pandemic, researchers have made significant efforts to develop innovative therapies and safe and effective vaccines to stop transmission of the virus and minimize COVID -19 mortality. Although several vaccines against COVID -19 have been approved and are in use, the COVID -19 pandemic has not yet been adequately eradicated. This is due to several challenges in rapid vaccination campaigns, such as difficulties in mass production and timely distribution of the appropriate doses to everyone worldwide. In addition, SARS-CoV-2 has a highly mutative genetic sequence that leads to the emergence of new mutant strains that compromise vaccine efficacy. Most currently approved antiviral drugs fail due to undesirable side effects and resistance. Therefore, there is an urgent need to discover and develop new mechanisms of action to treat COVID -19. One of the promising mechanisms is targeting host cell proteases instead of viral proteins. Host cell proteases facilitate the entry of SARS-CoV-2 through proteolytic cleavage and activation of the S protein, which is a necessary step for the host cell to be exposed to viral RNA. Since the viruses themselves cannot undergo cleavage to enter the host cell, they rely on host cell proteases to do so. The role of host cell proteases in SARS-CoV-2 entry makes them attractive targets to combat viral proliferation and minimize the outbreak of new variants. Inhibition of host cell proteases promises to target not only SARS-CoV-2 but also other respiratory viruses that have a similar entry process. However, host cell protease inhibitor development is not a simple process, as efficacy, selectivity, toxicity profiles, and treatment duration must be considered. Even though host cell proteases are stable and drug-effective targets, their experimental 3D crystal structures are not fully known. The disadvantage of current inhibitors is that they can disrupt normal physiological processes, which increases the likelihood of adverse effects and narrows the therapeutic window. Therefore, host cell protease inhibitors should be combined with viral protein inhibitors, as this approach should increase antiviral efficacy and minimize side effects at low doses. The discovery of potent and well-tolerated host protease inhibitors should improve the arsenal of antiviral drugs against current pandemics and future respiratory viruses.

LIST OF ABBREVIATION

FP	=	Fusion peptide
hACE2	=	Human angiotensin-converting enzyme 2
NTD	=	N-terminal domain
ORF	=	Open reading frames
RBD	=	Receptor-binding domain
RTC	=	Replicase transcriptase complex
TM	=	Transmembrane domain

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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