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Cathepsin L-selective inhibitors: A potentially promising treatment for COVID-19 patients

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ABSTRACT

The widespread coronavirus SARS-CoV-2 has already infected over 4 million people worldwide, with a death toll over 280,000. Current treatment of COVID-19 patients relies mainly on antiviral drugs lopinavir/ritonavir, arbidol, and remdesivir, the anti-malarial drugs hydroxychloroquine and chloroquine, and traditional Chinese medicine. There are over 2,118 on-going clinical trials underway, but to date none of these drugs have consistently proven effective. Cathepsin L (CatL) is an endosomal cysteine protease. It mediates the cleavage of the S1 subunit of the coronavirus surface spike glycoprotein. This cleavage is necessary for coronavirus entry into human host cells, virus and host cell endosome membrane fusion, and viral RNA release for next round of replication. Here we summarize data regarding seven CatL-selective inhibitors that block coronavirus entry into cultured host cells and provide a mechanism to block SARS-CoV-2 infection in humans. Given the rapid growth of the SARS-CoV-2-positive population worldwide, ready-to-use CatL inhibitors should be explored as a treatment option. We identify ten US FDA-approved drugs that have CatL inhibitory activity. We provide evidence that supports the combined use of serine protease and CatL inhibitors as a possibly safer and more effective therapy than other available therapeutics to block coronavirus host cell entry and intracellular replication, without compromising the immune system.

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1. Introduction

In early December of 2019, a novel coronavirus associated with atypical pneumonia emerged from Wuhan, China (Zhu et al., 2020). Over the past 5 months, it has affected over 84,000 individuals nationwide, affecting men and women from infants to seniors according to the situation report from the Centers for Disease Control and Prevention of the United States on May 12, 2020 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports). The severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) crystal structure and genomic sequence were published between January and February 2020 (Chan et al., 2020; Lu et al., 2020; Yan et al., 2020). This new coronavirus strain is an enveloped single-stranded RNA virus that appears as

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome-coronavirus-2; βCoV, betacoronavirus; MERS-CoV, Middle East respiratory syndrome-coronavirus; WHO, World Health Organization; COVID-19, coronavirus disease; CatL, cathepsin L; SI, selectivity index; HIV, human immunodeficiency virus; ChiCTR, Chinese Clinical Trial Registry; TMPRSS2, transmembrane serine protease 2; MW, molecule weight; S1, S2, spike protein subunits; RBD, receptor-binding domain; ACE2, angiotensin-converting enzyme 2; ATII, human lung type-II alveolar epithelial cells; SARS-S, SARS S protein; DEP, dual-envelop pseudotype; CPE, cytopathic effect; GFP, green fluorescent protein; CC50, the half cytotoxic concentration; IC50, the half maximal inhibitory concentration; EC50, the half effective concentration; FDA, Food and Drug Administration; CatS, cathepsin S; APC, antigen presenting cell; MRSA, Methicillin-resistant *Staphylococcus aureus*; ARDS, adult respiratory distress syndrome.

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round or oval particles with a diameter of 6–14 nm (Chan et al., 2020; Jin et al., 2020; Yan et al., 2020). It belongs to the betacoronavirus (βCoV) lineage, with surface spike proteins similar to the former known βCoVs, such as the HCoV-OC43, HCoVHKU1, SARS-CoV, and Middle East respiratory syndrome (MERS)-CoV (Zhu et al., 2020). The genomic sequence of this novel coronavirus shares 82% identity with that of SARS-CoV Tor2 (AY274119) (Chan et al., 2020; Morse, Lalonde, Xu, & Liu, 2020). Because of their genetic relatedness, the new coronavirus was initially named 2019-nCoV and shortly after it was renamed as SARS-CoV-2.

Distinct from other coronaviruses, SARS-CoV-2 shows high infectivity for humans with a secondary attack rate among close contact of 35% (Liu, Eggo, & Kucharski, 2020) *versus* 6.2% for SARS-CoV and 2.7–32.3% for MERS-CoV, respectively (Goh et al., 2004; Van Kerkhova et al., 2019). After the first patient was identified in December 2019 (Huang et al., 2020; Li et al., 2020), this virus spread rapidly from Wuhan to nearly all 34 provinces, municipalities, and special administrative regions in China and over 250 countries, territories, and areas around the globe (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports). As the numbers of cases continue to mount globally, the World Health Organization (WHO) identified the SARS-CoV-2 infection as an acute public health event on January 30th, 2020. On February 19th, 2020, the WHO named this SARS-CoV-2 infection in humans coronavirus disease "COVID-19." SARS-CoV-2 has a reported 3% mortality rate based on current public information and clinical observations (Zumla, Hui, Azhar, Memish, & Maeurer, 2020; WHO Director-General's opening remarks at the media briefing on COVID-19 - 3 March 2020 - World Health Organization, March 3, 2020). By May 12th, 2020, there were over 78,000 total reported deaths in the US and over 283,000 deaths worldwide (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports). At the onset of illness, most patients experience fever and fatigue, accompanied with dry cough (Chen et al., 2020). Some patients also showed few or no symptoms but were laboratory-confirmed positive. These patients are asymptomatic carriers who make the transmission extremely difficult to monitor and control (Rothe et al., 2020). Some patients develop dyspnea, multifocal pneumonitis that can cause a rapid decrease of blood oxygen saturation, and systemic cytokine storm, multisystem organ failure, and death (Chen et al., 2020).

Effective treatment of COVID-19 patients presents an urgent unmet need. While the world awaits the development of a protective vaccine for SARS-CoV-2, which the infection morbidity and associated death toll are still on the rise, the discovery of clinically effective SARS-CoV-2-specific drugs has been the focus of governments, research institutions, drug companies, and hospitals worldwide. We hereby call attention to a novel mechanism of cysteinyl cathepsin L (CatL) activity in coronavirus surface spike protein proteolysis and propose a promising possibility of a protease inhibitor cocktail therapy to target host cell surface transmembrane serine protease 2 (TMPRSS2) and CatL on cell surfaces and inside the endosomes.

Clinical trials and anti-viral drug candidates.

Since the outbreak of COVID-19 in China and then worldwide, the drug treatments offered to COVID-19 patients have shown inconsistent outcomes. Most drugs were administered based on the anti-coronavirus effects demonstrated in previous *in vitro* and patient studies.

1. Registered clinical trials.

Fig. 1 summarizes current registered COVID-19-associated trials through May 5, 2020 from various clinical trial registry sites. There are 2,118 trials in total and the majority of which are registered at ClinicalTrials.gov from the United States National Library of Medicine at the National Institutes of Health ($n = 1,076$) and from the Chinese Clinical Trial Registry (ChiCTR) database ($n = 653$) (Fig. 1a). Of these 2,118 trials, 1,273 were intervention studies to test the efficacy of medications with proper placebo or standard treatment controls. There are 756 observational trials in which researchers do not intervene but monitor participant disease progress for the purpose of information collection. The remaining registered trials include diagnostic tests, expanded access, epidemiological inquiry, health service research, basic science, prevention, meta-analysis, and studies of prognosis (Fig. 1b). Although most trials were "Not applicable" without defined phases, there were 824 trials covering from Phases I ~ IV. Others include 117 retrospective studies and 6 new treatment trials for small sample pilot studies (Fig. 1c). From all listed trials, 1,067 were involved in pharmacologic interventions whereas 1,051 concerned other medical interventions, such as mechanical ventilation, continuous renal replacement therapy, mental status evaluation, diagnostic studies, and biomarker research. Of the pharmacological intervention trials, 873 were controlled and 194 lacked control arms. Controlled trials included 697 randomized and 176 non-randomized studies (Fig. 1d).

The 1,067 medication intervention trials include 34 drugs, among which chloroquine and traditional Chinese medicine have the highest numbers of trials and patients (Table 1). Chloroquine, a small molecule anti-malarial agent, showed efficacy in inhibiting viral infection in cell assays (Wang, Cao et al., 2020). There are at least 180 clinical trials that are underway, containing 215,842 participants. Traditional Chinese medicines, such as houttuynia mixture with qingfei prescription, honeysuckle decoction, Ba-Bao-Dan, Tanreqing capsules, and Shenqi Fuzheng are included in 121 trials enrolling 59,562 patients. Of the 1,067 medication intervention trials (Fig. 1d), 12 phase-III randomized placebo-controlled trials of 13,465 mild/moderate to severe COVID-19 patients are assessing the efficacy of remdesivir, a small molecule weight (MW = 602.6) drug originally from Gilead Sciences (Foster City, California) that targets Ebola, Marburg, and MERS virus infections (Sheahan et al., 2020; Warren et al., 2016). There are 19 trials with 18,130 patients testing the efficacy of lopinavir/ritonavir, protease inhibitors originally developed from the Abbott Laboratories (Lake Bluff, Illinois) to treat patients with human immunodeficiency virus (HIV) infections (Corbett, Lim, & Kashuba, 2002). Arbidol (also called Umifenovir) is another small molecule drug that has been mostly used in China and Russia to target influenza RNA virus (Haviernik et al., 2018). A total of 10 trials and 3,177 patients are currently enrolled. Fourteen trials evaluate vaccine candidates in an aggregate of 10,325 participants. Some other major therapies include cytokine monoclonal antibody/inhibitors, convalescent plasma therapy, azithromycin and corticosteroids, as also listed in Table 1.

2. Top drug candidates.

The announced results from *in vitro* tests and clinical feedback from case studies and news reports suggest possible efficacy of at least 4 drugs, including remdesivir, chloroquine, lopinavir/ritonavir, and arbidol, although detailed clinical trial data are pending (Fig. 1 and Table 1). All these drugs have been reported previously to be effective in clinical use or *in vitro* tests against SARS-CoV or MERS-CoV (Agostini et al., 2018; Chu et al., 2004; Khamitov et al., 2008; Vincent et al., 2005).

Remdesivir, a small molecule adenosine analogue, was proved effective at the stage of post SARS-CoV-2 entry (Wang, Cao et al., 2020). Chloroquine, a 70-year-old anti-malarial and autoimmune disease drug, can block virus infection *in vitro* at both the entry and post-entry stages of SARS-CoV-2 infection (Wang, Cao et al., 2020). These two drugs worked at low μM concentrations and showed high selectivity indices (SI) in Vero E6 cells infected with SARS-CoV-2 (Wang, Cao et al., 2020). Arbidol, a broad-spectrum anti-influenza A/B drug, inhibited the activity of novel coronavirus at 10–30 μM concentrations from *in vitro* assays. Yet, many of these observations remain preliminary. Clinical reports have suggested a few drugs with possible efficacy for COVID-19 patients. A case report in the United States anecdotally indicated the effectiveness of remdesivir on a COVID-19 patient (Holshue et al., 2020). A recent cohort of 53 patients hospitalized for severe COVID-19 from the United States, Canada, and Japan showed 68% improvement in oxygen saturation but with a mortality rate of 18% after 10 days of remdesivir (Grein et al., 2020). Four patients with mild or severe COVID-19 pneumonia admitted to Shanghai Public Health Clinical Center showed

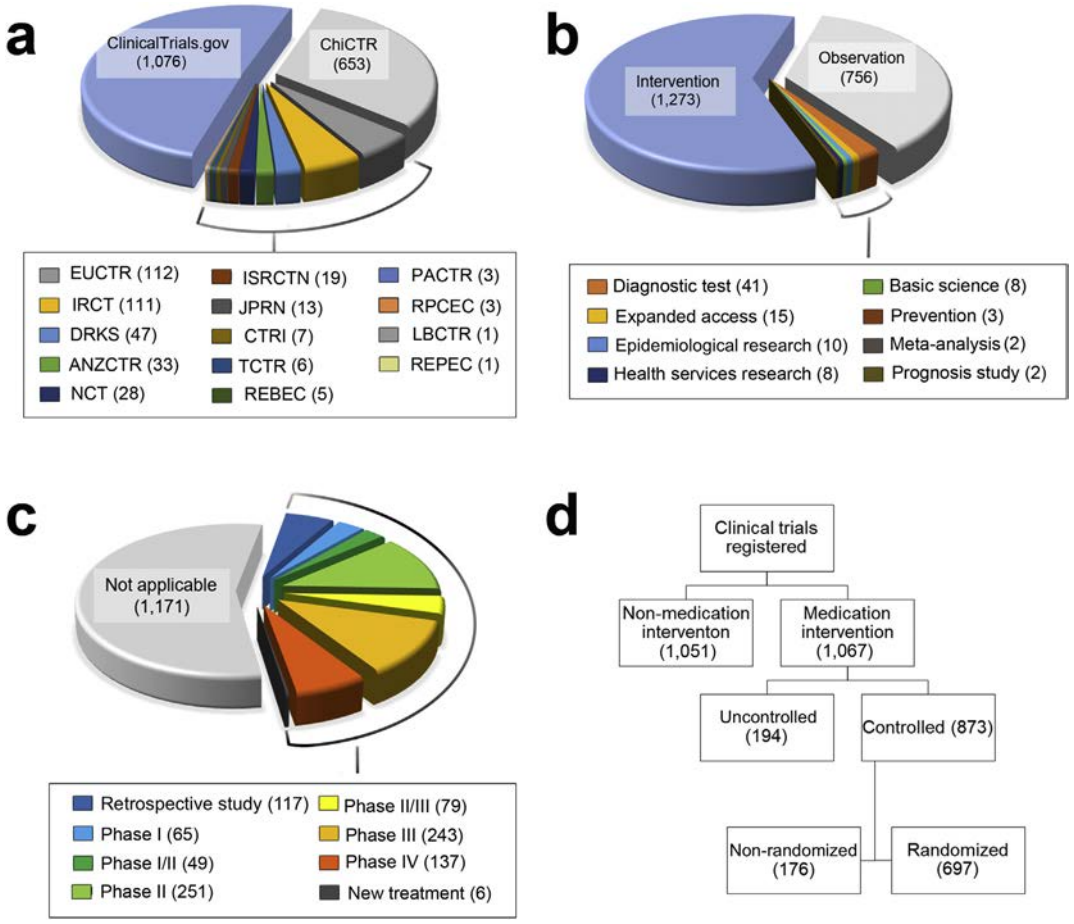

Fig. 1. Current registered clinical trials. **a.** International databases of registered clinical trials from ClinicalTrials.gov from the United States National Library of Medicine at the National Institutes of Health, Chinese Clinical Trial Registry (ChiCTR); European Union Clinical Trials Register (EUCTR); Iranian Registry of Clinical Trials (IRCT); German Clinical Trials Register (DRKS); Australian New Zealand Clinical Trials Registry (ANZCTR); Netherlands Clinical Trials (NCT); International Standard Randomized Controlled Trial Number (ISRCTN); Japan Primary Registries Network (JPRN); Clinical Trials Registry – India (CTRI); Thai Clinical Trials Registry (TCTR); Brazilian Clinical Trials Registry (REBEC); Pan African Clinical Trial Registry (PACTR); Cuban Public Registry of Clinical Trials (RPCEC); The Lebanese Clinical Trials Registry (LBCTR); and Peruvian Clinical Trials Registry (REPEC). **b.** Clinical trials sorted by study types. Most are intervention studies and observational studies. The remaining includes diagnostic test (image diagnosis, IgG and IgM test, nucleic acid or RNA test compared with the golden standard test); expanded access, sometimes called "compassionate use" (a potential pathway for a patient with an immediate life-threatening condition or serious disease or condition to gain access to an investigational medical product –drugs, biologics, or medical devices– for treatment outside of clinical trials when no comparable or satisfactory alternative therapy options are available); epidemiological research (mainly cross-sectional studies describing the clinical features of COVID-19, psychological status, or biomarkers); basic science (trials with detailed basic cellular and molecular studies of patients); health services research (evaluation of health service providers' mental status or protection measures); prevention study (evaluates the effectiveness of the medication or preventive protocols); Meta-analysis (statistical procedure for combining data from multiple studies); prognosis study (analyzes the clinical outcomes from study cohorts); and screening study (studies of patient CT scan results). **c.** Clinical trials sorted by different phases. Not applicable trials include those without phase information. Retrospective study includes mainly the case controls or case series studies based on dataset from medical record. New treatment includes studies for small sample pilot studies (such as rehabilitation, plasma therapy, or traditional Chinese medicine) that are mainly from ChiCTR. **d.** Clinical trials grouped by non-medication intervention and medication intervention that is further grouped as uncontrolled and controlled trials. Controlled trials include randomized and non-randomized trials.

significant improvement of symptoms after both the anti-viral treatment with lopinavir/ritonavir (Kaletra®), arbidol, and Shufeng Jiedu capsule (a traditional Chinese medicine) for 6–15 days together with antibiotics treatment and supplemental oxygen (Wang, Chen et al., 2020). Yet, a recent trial of 99 adults hospitalized with severe COVID-19 from China did not show significant clinical improvement after 14 days of lopinavir/ritonavir treatment compared with 100 patients with standard-care (Cao et al., 2020). No firm results are available regarding these drugs from larger cohorts of patient trials.

CatL mediates coronavirus infection and replication.

The high structural similarity between SARS-CoV-2 and SARS-CoV or MERS-CoV, and their similar clinical presentations suggest that SARS-CoV-2, SARS-CoV, and MERS-CoV will respond similarly to therapeutics targeting coronavirus spike protein processing. We review here related studies from the past 10 years and propose that CatL is an attractive therapeutic target to protect COVID-19 patients from host cell virus entry and intracellular virus replication, while leaving the host adaptive immunity unaffected.

1. Coronavirus infection and replication.

SARS-CoV-2 and most other coronaviruses, such as SARS-CoV, share similar viral structures and virulence mechanisms (Simmons, Zmora, Gierer, Heurich, & Pohlmann, 2013). The spike glycoprotein (S glycoprotein) on the coronavirus surface is synthesized as a ~ 1,300 amino acids precursor that consists of a ~ 700 amino acid N-terminal signal subunit (S1) (685 amino acids for SARS-CoV-2) and a ~ 600 amino acid C-terminal transmembrane subunit (S2) (588 amino acids for SARS-CoV-2) (Li, 2016). While the S1 subunit contains a receptor-binding domain (RBD) that mediates the binding of coronavirus on to the host cell surface receptor angiotensin-converting enzyme 2 (ACE2), the S2 contains a hydrophobic fusion peptide and two heptad repeat regions that mediate virus fusion with the host cells (Belouzard, Millet, Licitra, & Whittaker, 2012; Simmons et al., 2013; Song, Gui, Wang, & Xiang, 2018). Protein sequence analysis of the 71-amino acid receptor binding motif within the RBD domain in the S1 subunit indicates that SARS-CoV-2 and SARS-CoV share 58 amino acid identity and form congruent 3D structures, while the S2 subunit of SARS-CoV-2 shares 99% identity

Table 1
List of drugs from on-going clinical trials for COVID-19 patients (up to May 5th, 2020).

Drug list*	Drug name	Number of trials	Total patient size
1	Chloroquine**	180	215,842
2	Traditional Chinese medicine	121	59,562
3	Lopinavir/Ritonavir	19	18,130
4	Remdesivir	12	13,465
5	Cytokine mAb /Inhibitor**	79	13,087
6	Vitamin	26	11,940
7	Vaccine	14	10,325
8	Convalescent plasma	59	9,626
9	Azithromycin	15	8,747
10	Corticosteroids**	37	6,913
11	Anticoagulants**	16	5,468
12	Interferon**	16	5,450
13	Anti-microbial/antibiotics**	18	4,840
14	ACEI/ARB***	15	4,180
15	Arbidol	10	3,177
16	Diuretics**	2	2,474
17	Stem cells therapies**	53	2,449
18	Favipiravir	19	2,310
19	Herbs extraction	18	1,818
20	Ruxolitinib	14	1,423
21	Antifibrosis**	10	1,325
22	Camostat/nafamostat	5	1,324
23	Chlorpromazine	3	1,050
24	Recombinant human ACE2***	4	600
25	Imatinib	2	485
26	Thymosin	3	470
27	Antiviral medication**	4	440
28	Immunoglobulins**	8	413
29	Anti-hepatitis C	8	378
30	Immune cell therapy**	9	360
31	HIV protease inhibitors**	3	238
32	Statin	3	200
33	Fingolimod	2	70
34	Others**	260	62,663
	Total	1,067	401,451

*Drug list is sorted based on study patient size. **Notes: **Chloroquine:** hydroxychloroquine, chloroquine phosphate, chloroquine analog (GNS651); **Cytokine mAb/inhibitor:** IL6 monoclonal antibodies tocilizumab, siltuximab, sarilumab, and clazakizumab; complement component 5 inhibitors ravulizumab and eculizumab; PD-1 blocking antibody nivolumab; human granulocyte macrophage colony-stimulating factor receptor inhibitors mavrilimumab and gimsilumab; IL17A antagonist ixekizumab, IL1β antibody canakinumab; vascular endothelial-derived growth factor antibody bevacizumab; IL1 receptor antagonist anakinra; anti-C5a receptor antibody avdoralimab; and tumor necrosis factor-α inhibitor adalimumab; **Corticosteroids:**ciclesonide, budesonide, methylprednisolone, prednisone, and dexamethasone; **Anticoagulants**: low-molecular-weight heparin, recombinant tissue-plasminogen activator, and nebulized heparin sodium; **Interferons:** IFN-α1b Eye Drops, IFN-β1b, IFN-β1a, IFN atomization, IFN-α1b spray, recombinant super-compound IFN; IFN aerosol inhalation; **Anti-microbial/antibiotics:** doxycycline, carrimycin, povidone-iodine, and levamisole; **Diuretics:** thiazide and spironolactone; **Stem cells therapies:** stem cells therapy, mesenchymal stem cells, adult allogeneic bone marrow—derived mesenchymal stromal cells, allogenic adipose tissue-derived mesenchymal stem cells, dental pulp mesenchymal stem cells; **Antifibrosis:** nintedanib and pirfenidone; **Antiviral medications:** oseltamivir and baloxavir marboxil; **Immunoglobulins:** intravenous immunoglobulin G (IVIG: are sterile, purified IgG products manufactured from pooled human plasma and typically contain more than 95% unmodified IgG) and immunoglobulin from cured patients; **Immune cell therapy:** NK cells; mononuclear cells; umbilical cord blood cytokine-induced killer cells; **HIV protease inhibitors:** ritonavir and darunavir/cobicistat; **Others**: oral nutrition supplements, non-steroidal anti-inflammatory drugs, anti-hypertension drugs, T3 solution, et al. ***Abbreviations:** ACEI/ARB: angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers; ACE2: angiotensin-converting enzyme 2.

with SARS-CoV (Hoffmann, Kleine-Weber et al., 2020; Shang, Yang, Rao, & Rao, 2020; Yan et al., 2020). The entry of SARS-CoV-2 into human lung type-II alveolar (ATII) epithelial cells employs the host cell surface ACE2, as does SARS-CoV (Hoffmann, Kleine-Weber et al., 2020; Lu et al., 2020; Zou et al., 2020). Fig. 2 provides a simplified diagram of the SARS-CoV-2 infection pathway from initial cell surface ACE2 binding, endocytosis, membrane fusion, intracellular virus replication, to the release of newly packaged SARS-CoV-2.

2. TMPRSS2 and CatL in coronavirus infection.

The binding of coronaviruses onto the lung ATII cell surface ACE2 receptor may facilitate virus surface S1 subunit proteolysis by plasma membrane-bound serine protease TMPRSS2 and CatL (Fig. 3a) (Hoffmann, Kleine-Weber et al., 2020; Liu et al., 2018; Zhang et al., 2019). This process may continue during virus endocytosis (Fig. 3b) (Hu, Dammer, Ren, & Wang, 2015; Wang et al., 2008). The serine protease TMPRSS2 functions at neutral pH (Meyer et al., 2013), but loses its activity under acidic conditions. We recently reported that CatL complexes with TGF-β receptor-1 on the kidney epithelial cell surface, a function of CatL that does not depend on its proteolytic activity (Zhang et al., 2019). Therefore, TMPRSS2 may play a major role in virus S1 subunit proteolysis on the host cell surface, although membrane-bound or released CatL should also target the same substrate (Zhang et al., 2019).

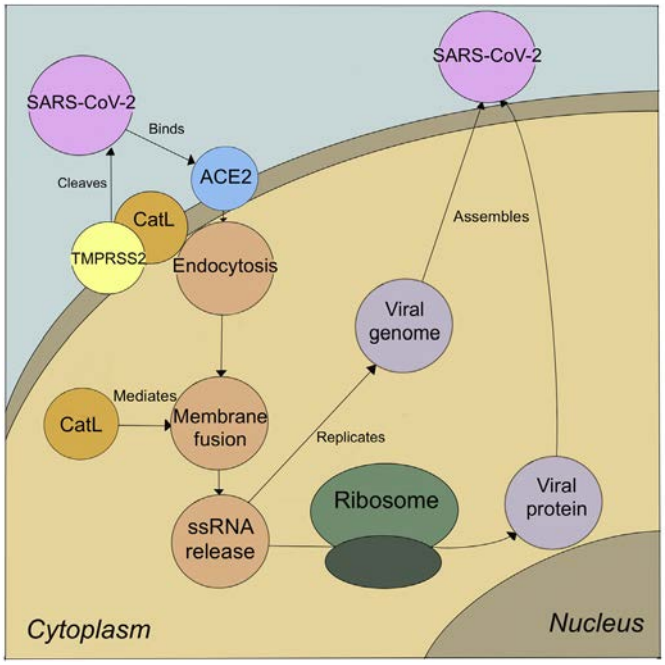

Fig. 2. Diagram of the lung type-II alveolar epithelial cell (ATII) SARS-CoV-2 infection pathway. This pathway includes initial SARS-CoV-2 surface spike protein proteolysis by ATII cell surface TMPRSS2 and CatL and then binding of SARS-CoV-2 to ATII cell surface receptor ACE2, followed by endocytosis. Membrane fusion occurs between the virus containing vesicles and endosome, resulting virus delivery into the endosomes where CatL cleaves the S1 subunits. The remaining S2 subunit on virus surface mediates virus fusion with the endosome membrane, leading to virus ssRNA release into the cytosol, where the virus replicates and directs protein synthesis (*via* host ribosomes), and the progeny virions assemble, acquire their coat, and exit the host cell to propagate infection to healthy cells.

3. CatL activity in coronavirus replication.

Once the SARS-CoV-2 reaches intracellular endosomes however, CatL becomes the major protease that cleaves the virus S1 subunit as this cysteinyl proteinase has an acidic pH optimum (Fig. 3c) (Chapman, Riese, & Shi, 1997; Ou et al., 2020). Indeed, SARS-CoV viruses have been considered pH-sensitive viruses and their intracellular trafficking requires an acidic environment (Chu, McElroy, Chu, Bauman, & Whittaker, 2006). While the serine protease TMPRSS2 acts locally at the host cell plasma membrane and possibly during endocytotic vesicle trafficking (Glowacka et al., 2011), CatL continues S1 subunit degradation in the acidic endosome and lysosome compartments. This sequence of events explains the observations that the TMPRSS2 inhibitors camostat mesylate and nafamostat mesylate, or a non-selective cysteinyl cathepsin inhibitor E64d, could partially limit the SARS-CoV and SARS-CoV-2 infection of human epithelial cells (Hoffmann, Kleine-Weber et al., 2020; Hoffmann, Schroeder et al., 2020). Combined use of camostat and E64d fully blocked the infection of these coronaviruses (Hoffmann, Kleine-Weber et al., 2020). Although not tested in Hofmann's studies, we hypothesize that combined use of

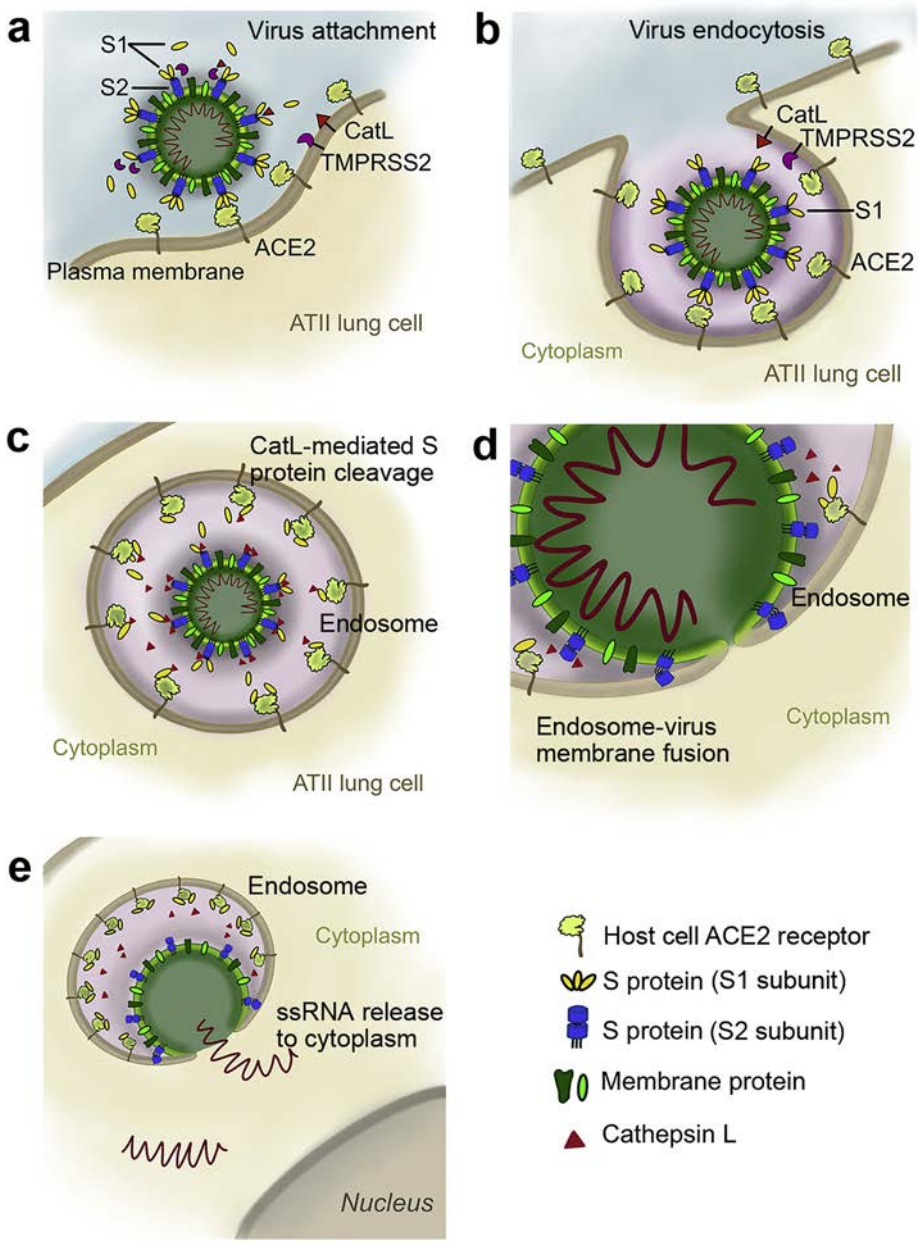

Fig. 3. Possible mechanism of CatL activity in mediating SARS-CoV-2 infection and replication. **a.** SARS-CoV-2 binding between the S protein S1 subunits and the cell surface receptor ACE2. **b.** Plasma membrane endocytosis together with the SARS-CoV-2. In these newly formed vesicles, SARS-CoV-2 remains attached to ACE2. **c.** SARS-CoV-2 targets to the endosomes where CatL cleaves the S1 subunits and frees the virus. **d.** Virus membrane fusion with the endosome membrane, leaving a gap at the fusion site. **e.** Endosome releases virus ssRNA into the cytosol where virus RNA produces viral proteins and packages new virus particles to release for next round of infections.

TMPRSS2 inhibitor camostat mesylate or nafamostat mesylate and a CatL inhibitor will blunt substantially coronavirus infection of human cells.

Human cysteinyl cathepsins are the major proteases that reside in endosomes. This family composes 11 members that display acidic pH optima (Liu et al., 2018). Among this family of proteases, CatL cleaves the virus spike glycoprotein at the position of T678 (VAYT-M) (Bosch, Bartelink, & Rottier, 2008), close to the predicted S1 and S2 boundary region of the SARS S protein (SARS-S) (Fig. 3c). After cleavage, the SARS-S S1 subunit is released from the S2 subunit. A fusion peptide on the S2 subunit inserts into the endosome membrane. Then the heptad repeats in the S2 subunit fold back and form a six-helix-bundle structure (Simmons et al., 2013). Subsequently, viral and endosome membranes coalesce and eventually fuse (Fig. 3d). Coronaviridae then release their RNA into the cytoplasm of the host cells (Fig. 3e). As a single-stranded, positive sense RNA virus, coronavirus has its own genetic material. The viral RNA can function as messenger RNA, directing the synthesis of viral proteins by host cell ribosomes without entering the nucleus (Fig. 2) (Positive stranded RNA virus replication. ViralZone. https://viralzone.expasy.org/1116). Therefore, CatL inhibition provides two sequential blocks for coronavirus infection: on the host cell surface to block virus entry and inside the host cell endosomes to block viral material release and replication. Before we discuss the potential advantage of CatL inhibition in maintaining human adaptive immunity, we will discuss the status of current CatL inhibitory drug development.

CatL inhibitors possess anti-coronavirus activity.

Based on the mechanisms proposed in Figs. 2 and 3, a CatL-inhibitory molecule can exert substantial anti-viral activity. Several compounds display CatL inhibitory activity, as summarized in earlier reviews (Li, Fang, & Ao, 2017). Yet, no currently available drug can specifically

inhibit CatL (Dana & Pathak, 2020). In 2003, the outbreak of SARS brought academic attention to CatL inhibitor development. Over the past decade, seven CatL inhibitory compounds have demonstrated anti-coronavirus activity. These compounds include dec-RVKR-CMK, K11777, small molecule 5705213, MDL28170, SSAA09E1, EST, and oxocarbazate. Table 2 lists these compounds, their development approach, tested viruses, and brief statements of outcomes.

1. CatL inhibitor pharmacology and toxicity.

All seven CatL inhibitors have been tested on the infection of coronavirus pseudotypes and demonstrated inhibition of virus entry into host cells, although their pharmacology and toxicity varied. K11777 showed the lowest IC50 in SARS infection inhibition (Zhou et al., 2015) among all 7 listed inhibitors in Table 2, although not all assays were performed in a single study. K11777, a vinyl sulfone cysteine protease inhibitor selected from screening of a library of proximately 2100 cysteine protease inhibitors using the dual-envelop pseudotype (DEP) assay (Zhou et al., 2015). It exhibited inhibition of SARS-CoV infection at IC50 of 0.68 nM, with CC50 > 10 μM and MERS-CoV infection at IC50 of 46 nM in the cytopathic effect (CPE) inhibition assay. Three potent K11777 analogs, SMDC256122, SMDC256159 and SMDC256160 also showed strong anti-viral activity towards SARS-CoV pseudotypes with IC50 at 0.04 nM, 0.07 nM, and 0.08 nM, respectively. The anti-viral infection activity of K11777, SMDC256159, and SMDC256160 was validated on replication of competent SARS-CoV at IC50 < 0.05 ± 0 nM, CC50 > 105.6 ± 59.3 μM, SI > 2112; IC50 0.65 ± 0.81 nM, CC50 > 109.2 ± 49.8 μM, SI > 168; IC50 < 0.08 ± 0.05 nM, CC50 = 50.6 ± 26.7 μM, SI > 632.5, respectively. Furthermore, K11777 and its analogs have already proven safe in several parasitic infections in animals, compatible with the feasibility of K11777 for clinical uses (Doyle, Zhou, Engel, & McKerrow, 2007; Engel, Doyle, Hsieh, & McKerrow, 1998).

The other six compounds possessed various potencies as virus infection inhibitor and for cytotoxicity. The most recently studied dec-RVKR-CMK showed inhibitory effects on MERS-CoV entry at noncytotoxic concentrations (2.5 to 100 μM) (Matsuyama et al., 2018; Millet and Whittaker, 2014), with a decrease in the number of green fluorescent protein (GFP)-positive cells by 60% with VSV-ΔG/GFP-MERS-S infection, tested in human epithelial cell lines LoVo cells and Calu-3 cells. A high-throughput library (Chembridge Diverset Library) screening using self-synthesized virus peptides identified a small molecule compound 5705213 (Elshabrawy et al., 2014). It showed a relatively weaker capacity on SARS-CoV (IC50 = 9.0 μM) entry into the host 293FT cells compared with K11777. Although it showed dose-dependent inhibition of SARS-CoV entry and the CC50 was of 400 μM, leading to a low SI (CC_{50}/IC_{50}) of 26.7. SSAA09E1, selected from the Maybridge Hitfinder chemical library using the SARS/HIV-luc pseudotyped virus infection assay, exerted inhibition on virus entry of ACE2-expressing 293 T cells at EC50 of 6.7 ± 0.4 μM, CC50 > 100 μM, SI > 16 (Adedeji et al., 2013). EST was reported as a cysteine protease inhibitor, including CatL. It showed strong inhibition on SARS-CoV entry when administered together with serine protease inhibitors, such as camostat mesylate (Kawase, Shirato, van der Hoek, Taguchi, & Matsuyama, 2012). Oxocarbazate (PubChem CID 23631927) was initially designed based on the knowledge of the previously reported thiocarbazate chemotype (Myers, Shah, Diamond, Huryn, & Smith 3rd., 2008; Shah et al., 2010). It inhibited SARS-CoV infection at IC50 of 273 ± 49 nM. In cultured human aortic endothelial cells, oxocarbazate did not display cytotoxicity at up to 100 μM (Shah et al., 2010). MDL28170, also known as calpain inhibitor-III or *Z*-Val-Phe-CHO, was first reported in 2005 (Simmons et al., 2005). It inhibited the infection of SARS-CoV pseudovirus at IC95 of 2.0 μM.

Detailed CatL inhibition efficacy information is only available from three of the seven compounds listed in Table 2. MDL28170 was selected from a library of pharmacologically active compounds. It showed potent CatL inhibition at IC50 of 2.5 nM (Simmons et al., 2005). Oxocarbazate (PubChem CID 23631927), originally selected from a CatL assay in 2010, inhibits CatL activity at IC50 around 6.9 nM and acts as a slow-on, slow-off inhibitor (Shah et al., 2010). SSAA09E1 was initially reported in 2013 to inhibit CatL activity with an IC50 of 5.33 ± 0.61 μM (Adedeji et al., 2013). Results showed that oxocarbazate had a similar inhibitory potency to MDL28170, but 1,000-fold more potent than SSAA09E1. Although no specific IC value was obtained from K11777, it was considered the most potent CatL inhibitor among the screened protease inhibitors (Zhou et al., 2015). The other 3 compounds (dec-RVKR-CMK, EST, and 5705213) were also selected from validated CatL inhibitors, but their specific potencies remained incompletely reported.

2. CatL inhibitor drug candidates.

The pharmacological details in Table 2 identify K11777 and oxocarbazate as the most attractive candidates for anti-coronavirus drugs. The available potency and safety assays identify CatL inhibitor K11777 as an efficacious drug candidate. Human preclinical trials are underway for K11777 as a potential treatment for patients with Chagas disease (Chaparro et al., 2018), and the dose ranging and safety data from these trials may greatly shorten the preclinical and drug safety test time towards a novel therapeutic agent for COVID-19 patients. However, K11777 is an irreversible covalent inhibitor of CatL, and this irreversibility often entails unacceptable toxicity. In contrast, oxocarbazate is a reversible inhibitor of CatL and acts at a low concentration (Shah et al., 2010), which meets the requirement for a potent, selective, and low toxicity candidate for human use. Detailed assays of oxocarbazate's activity against SARS-CoV-2 and relevant animal experiments should be conducted urgently.

FDA-approved drugs targeting CatL in coronavirus infection.

Although several potential therapeutic CatL inhibitor candidates exist to date, such as K11777 and oxocarbazate, the ubiquitous expression of CatL raises concern for unwanted adverse effects of CatL inhibition. Therefore, we examined the inventory of the United States Food and Drug Administration (FDA)-approved drugs that may be effective in treating SARS-CoV-2 infections, and listed ten currently FDA-approved drugs that exhibit CatL inhibitory activity in Table 3, in which we sorted the list of drugs by type, including antimicrobial, antimalarial, immunomodulatory agents, and others. These already approved drugs may be redeployed to treat SARS-CoV-2 infection.

1. Antimicrobial drugs.

Several antimicrobial drugs inhibit CatL activity in human cells. Furthermore, these antimicrobial drugs can be classified as antibiotics (teicoplanin), anti-tuberculous drugs (rifampicin), anti-leprosy (clofazimine) and anti-HIV agents (saquinavir). Glycopeptide drugs such as teicoplanin can potently inhibit CatL activity in a dose-dependent manner (Zhou et al., 2016). These drugs are usually used in the clinic to treat Gram-positive bacteria, especially Methicillin-resistant *Staphylococcus aureus* (*S. aureus*, MRSA) and *S. pneumoniae*, with low toxicity and relatively low safety concern. Bacterial superinfection commonly complicates viral pneumonitis, particularly in patients who require endotracheal intubation (Chertow & Memoli, 2013; Rice et al., 2012). The teicoplanin family glycopeptide antibiotics merit particular consideration in this situation due to their anti-CatL activity. Notably, glycopeptide drugs such as teicoplanin can inhibit the infection of Ebola, MERS, SARS and SARS-CoV-2 viruses by inhibiting CatL (Zhang et al., 2020; Zhou et al., 2016). A recent study from China published in bioRxiv suggested that teicoplanin can block SARS-CoV-2 infection in a dose-dependent manner in A549 cells, HEK293 T cells, and huh7 cells (Zhang et al., 2020). These encouraging findings support our hypothesis that targeting CatL can treat SARS-CoV-2 infection. Rifampicin and clofazimine inhibited CatL competitively or non-competitively, respectively (Kamboj et al., 2003). Saquinavir prevented vascular damage by inhibiting CatL activity (Cai et al., 2017).

2. Antimalarial drugs.

The antimalarial drug, chloroquine can effectively block SARS-CoV-2 infection in cultured cells (Wang, Cao et al., 2020). This drug inhibits lysosomal cathepsins nonspecifically by increasing endosomal pH (Tang et al., 2018; Shivanna, Kim, & Chang, 2014; Wang, Cao et al., 2020;

Table 2
Coronavirus effective cathepsin L inhibitor compounds.

No (ref)	Molecule	Explore approach	Virus	Pseudotypes	Cell type	Function	Outcome
1 (Matsuyama et al., 2018; Millet and Whittaker, 2014)	Ddec-RVKR-CMK (ecanoyl-Arg-Val-Lys-Arg-chloromethylketone)	Not mentioned	MERS-CoV	VSV-based pseudotyped virus bearing MERS-CoV S protein with GFP or Luc; authentic MERS-CoV	Vero TMPRSS2	Inhibits CatL activity	High concentration (100 μM) of dec-RVKR-CMK completely suppressed CatL and CatB.
						Inhibits virus entry	GFP-positive cells reduced by 60% after VSV-ΔG/GFP-MERS-S infection; and 40% (0.21 log) after VSV-ΔG/Luc-MERS-S infection. Infection by authentic MERS-CoV leads to a 97% reduction in viral mRNA copy number.
						Safety	Non-cytotoxic concentrations (2.5 to 100 μM) of dec-RVKR-CMK prevents entry of pseudotyped and authentic MERS-CoV.
2 (Kawase et al., 2012)	EST [(23,25)trans-epoxysuccinyl-l-leucylamindo-3-methylbutane ethyl ester]	Not mentioned	SARS-CoV	VSV-based pseudotyped virus bearing SARS-CoV S protein	293 T cells	Inhibits virus entry	Inhibits pseudotyped SARS-S infection of TMPRSS2-negative cells by ~80% by CatL inhibitor-III (219427 from Calbiochem). Inhibits pseudotyped SARS-S infection of TMPRSS2-expressing cells by 30–40% in the presence of cathepsin inhibitors.
						Safety	Not mentioned.
3 (Zhou et al., 2015)	K11777 (Vinylsulfone cysteine protease inhibitors) ((2S)-N-[(1E,3S)-1-(benzenesulfonyl)-5-phenylpent-1-en-3-yl]-2-{[(E)-4-methylpiperazine-1-carbonyl] amino}-3-phenylpropanamide)	Screened a library of ~2100 cysteine protease inhibitors with confirmed activity against human cathepsins, using dual-envelope pseudotype assays	SARS-CoV, EBOV	HIV-based pseudotypes bearing spikes from coronaviruses (SARS-CoV, HCoV-229E, NL63, MERS-CoV) or glycoproteins from filoviruses (EBOV, SUDV, TAFV, RESTV, BEBOV and MARV)	293 T, clone 17 express ACE2 (293 T/ACE2), 293 express human CD13 (293/CD13), Vero, and Huh7.5	Inhibits CatL activity	K11777 shows the most robust activity among the screened protease inhibitors. It inhibits a variety of cysteine proteases, including human cysteinyl cathepsins and cathepsin-like proteases from several other parasites.
						Inhibits virus pseudotype infection and toxicity (K11777 and its analogs)	Virus strain: Urbani; Assay: cytopathic effect inhibition (CPE). K11777 (IC50 = 0.68 nM, CC50 > 10 μM for SARS; IC50 = 46 nM for MERS-CoV); SMDC256122 (SARS-CoV IC50 = 0.04 nM); SMDC256159 (SARS-CoV IC50 = 0.07 nM); SMDC256160 (SARS-CoV IC50 = 0.08 nM).
						Inhibits competent SARS-CoV infection, replication and toxicity (K11777 and its analogs)	K11777 (SARS-CoV IC50 < 0.05 ± 0 μM, CC50 > 105.6 ± 59.3 μM; SI > 2112); SMDC256159 (IC50 0.65 ± 0.81 μM, CC50 > 109.2 ± 49.8 μM;

(continued on next page)

Table 2 (*continued*)

No (ref)	Molecule	Explore approach	Virus	Pseudotypes	Cell type	Function	Outcome
							SI > 168); SMDC256160 (IC50 < 0.08 ± 0.05 μM, CC50 = 50.6 ± 26.7 μM; SI > 632.5).
						Safety	It was already in advanced stages of development for several parasitic diseases and is safe and effective in animal models.
4 (Bertram et al., 2013; Simmons et al., 2005)	MDL-28170 (calpain inhibitor III, or *Z*-Val-Phe-CHO)	Not mentioned	HCoV-229E	HIV-1-derived vectopseudotyped with 229E-S	293 T cells	Inhibits virus infection	The TMPRSS2/serine protease and CatL pathways are both operational in Caco-2 cells. A combination of camostat and MDL-28170 is required to reduce transduction to background levels.
		High throughput screening for CatL inhibitors	SARS-CoV	HIV (SARS-S) pseudovirions	293 T cells transient expression of human ACE2 (293 T/ACE2)	Inhibits virus entry	IC50 = 2.5 nM; IC95 = 2.0 μM.
						Safety	Not mentioned.
5 (Elshabrawy et al., 2014)	Small molecule 5705213 {methyl-N-[4,6-bis(isopropylamino)-1,3, 5-triazin-2-yl]-N-cyanoglycinate} and derivative 7402683 {methyl-N-[4-(tert-butylamino)-6-(ethylamino) -1,3,5-triazin-2-yl]-N-cyanoglycinate}	High-throughput screening assay – fluorescence resonance energy transfer-based assay using self-synthesized virus peptides	SARS-CoV	pHIV-GFP-luc expression vector-bearing SARS-CoV-S	293FT transiently expression of human ACE2	Inhibits virus infection	5705213: IC50 = 9 μM for SARS-Cov-s; CC50 = 400 μM;SI (CC50/IC50) = 26.7; 5705213 and 7402683: dose-dependently inhibit CatL cleavage of the recombinant SARS-CoV-S. 5705213 + TMPRSS2 inhibitor show enhanced activity to inhibit SARS-CoV-S pseudotyped virus entry.
						Safety	Did not show significant cytotoxic effect on the 293FT cells at 10–100 μM concentrations.
6 (Shah et al., 2010)	Oxocarbazate (N -[(*S*)-2-tertbutoxy carbonylamino-3-(1H-indol-3-yl)-propionyl]-hydrazine carboxylic acid 2-(3,4-dihydro-2H-quinolin-1-yl)-2-oxo-ethyl ester)	High throughput screening for CatL inhibitors	SARS-CoV	HIV-luciferase vector, pNL-luc or SARS Spike proteins	293 T cells	Inhibits CatL activity	Time-dependent inhibition at IC50 from 6.9 ± 1.0 nM (immediately) to 2.3 ± 0.1 nM (1 h) to 1.2 ± 0.1 nM (2 h) to 0.4 ± 0.1 nM (4 h); CatL/CatB selectivity ratio:735.
						Inhibits virus entry	SARS-CoV: IC50 = 273 ± 49 nM.
						Safety	Nontoxic to human aortic endothelial cells up to 100 μM.
7 (Adedeji et al., 2013)	SSAA09E1 {[(Z)-1-thiophen-2-ylethylideneamino] thiourea}	Screening of a library of pharmacologically active small molecules using SARS/HIV pseudotyped virus infection assay	SARS-Cov	HIV-1 pseudotyped with SARS-CoV surface glycoprotein S (SARS-S)	293 T cells	Inhibits CatL activity	IC50 = 5.33 ± 0.61 μM
						Inhibits virus entry	EC50 = 6.7 ± 0.4 μM; CC50 > 100 μM; SI >16.
						Safety	Not mentioned.

Porotto et al., 2009). Chloroquine also affects the CDP/Cux transcription factor at neutral pH, indicating that it can directly affect protease synthesis (Goulet et al., 2004). Although chloroquine could have short-term benefit to COVID-19 patients by inhibiting CatL activity, it remains unproven, and can predispose to cardiac arrhythmia (Tonnesmann, Kandolf, & Lewalter, 2013). Non-selective inhibition of endosomal proteases, including cathepsins may cause unwanted effects in patients, as we will discuss further.

3. Immunomodulatory drugs.

Some immunomodulatory drugs have a proven role in inhibiting CatL. Dexamethasone inhibits CatL in muscle cells (Nguyen-Ba Robert, Dhalluin, Tapiero, & Hornebeck, 1994; Crossland, Constantin-Teodosiu, Greenhaff, & Gardiner, 2010). Astaxanthin, a potential immunomodulatory antioxidant agent, can suppress CatL activity in both Syrian hamster embryo cells and muscle cells (Shibaguchi et al., 2016). Many individuals with advanced SARS-CoV-2 infection have cytokine storm, often a harbinger of fatal outcome. This uncontrolled elevation of cytokines can lead to disseminated intravascular coagulation and multiple organ system failure (Chertow & Memoli, 2013; Rice et al., 2012). In survivors, the long-term consequences of cytokine storm may lead to pulmonary fibrosis, causing functional disability and reduction in quality of life (Chertow & Memoli, 2013; Rice et al., 2012). Interrupting this exaggerated inflammatory response should be a priority. To this end, trials discussed in Table 1 are evaluating anti-cytokine therapy, glucocorticoids, and interferon-α treatment for COVID-19-infected patients with adult respiratory distress syndrome (ARDS) and cytokine storm.

4. Others.

Clenbuterol and heparin weakly inhibit CatL and may have adjunctive value in certain situations in COVID-19 disease. Pneumonia can induce bronchospasm (Goncalves et al., 2012; Higgins, Fox, Kowalski, Nielsen, & Worrall, 2010). Clenbuterol, an inhaled selective 2-adrenergic agonist can limit CatL mRNA levels (Goncalves et al., 2012). COVID-19 patients have a high risk of deep vein thrombosis and those in need of anticoagulant therapy, heparin merits consideration as it may accelerate CatL inhibition by serpins (Higgins et al., 2010). Although not US FDA-approved, some Chinese medicine extracts can also inhibit CatL activity and broadly used among patients. MOL736, also called aurantiamide acetate that inhibits CatL activity, may relieve cough and reduce sputum production (Wang et al., 2007). The water and ethanol extracts of drynariae rhizoma also showed significant CatL inhibitory activities (Jeong et al., 2004). Numerous clinical trials underway will evaluate the therapeutic efficacy of these traditional Chinese medicines on COVID-19 (Table 1). Those preparations that exhibit CatL inhibitory properties may have particular potential as drug candidates for COVID-19.

Protease inhibitor cocktail therapy for COVID-19.

Development and validation of a therapy for COVID-19 presents a major and imminent challenge to society and medicine. Although several drugs show efficacy in inhibiting SARS-CoV-2 replication or infectious activity *in vitro*, clinical trials will require many months. Compassionate use of remdesivir in the first confirmed case in the United States and a recent study of 53 patients showed possible efficacy (Grein et al., 2020; Holshue et al., 2020), but the clinical effectiveness and safety of such agents require validation in rigorous controlled clinical trials.

In 2013, Bertram et al. proved that CatL-selective inhibitor MDL-28170 (Table 2) completely blocked the entry of HCoV-229E when the same dose (10 μM) of MDL-28170 was used together with 1 μM of TMPRSS2 inhibitor camostat mesylate, equivalent or significantly more potent than the inhibitory activity from 100 μM camostat mesylate alone depending on the host cell types (Bertram et al., 2013), suggesting a synergic role of CatL and TMPRSS2 in cellular penetration of coronavirus HCoV-229E (Fig. 3a). Recent reports tested the efficacy of TMPRSS2 inhibitors camostat mesylate and nafamostat mesylate in SARS-CoV and SARS-CoV-2 infection (Hoffmann, Kleine-Weber et al., 2020; Hoffmann, Schroeder et al., 2020). Camostat mesylate and nafamostat mesylate reduced the entry of these viruses into host cells that express TMPRSS2, and the pan-cathepsin inhibitor E64d blocked virus entry independent of TMPRSS2 expression. Again, combined use of E64d and camostat mesylate showed complete inhibition of SARS-CoV and SARS-CoV-2 entry into either TMPRSS2-positive or negative host cells (Hoffmann, Kleine-Weber et al., 2020). Although none of these studies used camostat mesylate or nafamostat mesylate combined with a CatL inhibitor in studying specifically SARS-CoV-2 infection, these prior findings provide encouragement with regard to COVID-19 treatment. First, camostat mesylate and nafamostat mesylate have been successfully and safely used to treat patients with chronic pancreatitis in Japan, and a randomized controlled trial has recently been completed in the United States (Ramsey, Nuttall, Hart, & Team, 2019). Use of these market drugs will help offset our potential side effect or toxicity concerns. Second, SARS-CoV-2 infection not only infects the respiratory tract, but also affects other organs in which not all cells express TMPRSS2 (Hoffmann, Kleine-Weber et al., 2020; Sungnak et al., 2020). A protease inhibitor cocktail approach using camostat mesylate or nafamostat mesylate together with a CatL inhibitor listed in Table 2 or even an FDA-approved CatL inhibitory drug listed in Table 3 might offer clinical improvement for COVID-19 patients by not only preventing lung epithelial cell infection, but also reducing the risks of SARS-CoV-2-induced damage in other cell types and organs with even much lower doses for each inhibitor than using camostat mesylate, nafamostat mesylate, or CatL inhibitor alone.

Protease inhibitor cocktail *versus* chloroquine.

While the combined use of TMPRSS2 inhibitor camostat mesylate and hydroxychloroquine has been included in two clinical trials (NCT04355052, NCT04338906), CatL inhibition should merit immediate consideration. Here we discuss the advantages of CatL inhibitor and camostat mesylate or nafamostat mesylate dual therapy *versus* camostat mesylate or nafamostat mesylate or CatL inhibitor monotherapy, and potential concerns of chloroquine treatment. The known molecular and cellular mechanisms of each of these drugs will help interpret the observations for the upcoming clinical data from the 4 on-going camostat mesylate trials (NCT04355052, NCT04338906, NCT04353284, NCT04321096), one on-going nafamostat mesylate trial (NCT04352400), and 180 on-going chloroquine trials (Table 1) and underscore the rationale of our proposed TMPRSS2 and CatL inhibitor cocktail therapy.

1. Three beneficial functions of dual protease inhibitor therapy.

We propose three beneficial functions of CatL inhibition in SARS-CoV-2 infections: blocking the virus entry on the host cell surface together with TMPRSS2 serine protease inhibition (*e.g.* camostat mesylate and nafamostat mesylate) (Fig. 3a), blocking virus membrane fusion in the endosome essential for release of the virion's genetic material and replication (Fig. 3c-3e), while not interfering with other essential protease activities of normal immune responses such as T-cell activation and anti-viral antibody production. When endosomal CatL activity in thymocytes and splenocytes is required for $CD4^+$ positive selection (Honey, Nakagawa, Peters, & Rudensky, 2002; Nakagawa et al., 1998), cathepsin S (CatS) is the major endosomal protease responsible for peripheral immune responses and antibody production (Beers et al., 2005; Riese et al., 1996; Shi et al., 1999). The proposed dual protease inhibitor therapy could combat SARS-CoV-2 infections not only at the entry point on the plasma membrane of the host cells, but also in the endosome, serial steps in viral pathogenesis in addition to preserving adaptive immunity.

2. Chloroquine nonspecifically impairs epithelial cell immunity.

Recent rudimentary clinical evidence suggested the efficacy of antimalarial chloroquine and its analogs hydroxychloroquine and chloroquine phosphate in COVID-19 patients. To date no registered clinical trial has supported this conjecture. Chloroquine affects ACE2 terminal glycosylation (Vincent et al., 2005) which affects in turn coronavirus host cell attachment. This mechanism could contribute to the reported efficacy of chloroquine (hydroxychloroquine or chloroquine

Table 3
A list of FDA-approved drugs that have CatL inhibitory activity.

No*	Drugs	Catagory	Function	Potential side effect	Ref.
1	Clofazimine	Antileprotic drugs	Inhibits cathepsin activities in a non-competitive manner with Ki of 0.25 mM.	Digestive symptoms; dry skin and discoloration (from pink to brownish-black) of the skin, stools, urine, saliva, sweat, tears or lining of the eyelids.	Kamboj et al., 2003
2	Glycopeptide antibiotics (Teicoplanin, dalbavancin, oritavancin, telavancin)	Antibiotics	Teicoplanin inhibits CatL activity in a dose-dependent manner and inhibits the entry of Ebola, MERS, and SARS viruses. Teicoplanin inhibits the entrance of SARS-CoV-2 spike-pseudoviruses into the cytoplasm in a dose dependent manner, with an IC50 of 1.66 μM.	Red man syndrome; nephrotoxicity including kidney failure and interstitial nephritis; neutropenia; deafness; QTc prolongation.	Zhou et al., 2016; Zhang et al., 2020
3	Rifampicin	Antituberculous	Inhibits CatL in a competitive manner with Ki of 0.125 mM.	Liver or kidney dysfunction; digestive symptoms.	Kamboj et al., 2003
4	Saquinavir (SQV)	Anti-HIV drug	Blocks recombinant mouse CatL activity *in vitro* and prevents intimal hyperplasia after arterial injury.	Digestive symptoms; may increase blood sugar levels and cause or worsen diabetes.	Cai et al. 2017
5	Chloroquine	Anti-malarial drug	>1 μM Chloroquine abolishes F protein proteolytic processing by inhibiting cathepsin activity because of pH changes. Inhibits CatL-mediated processing of the CDP/Cux transcription factor at a neutral pH by inhibiting the cathepsin activities.	Liver dysfunction; digestive symptoms; pancytopenia; aplastic anemia; reversible agranulocytosis; low blood platelets; neutropenia.	Porotto et al., 2009; Goulet et al., 2004
6	Astaxanthin	Antioxidant agent	In the Astaxanthin group mouse, 10 days of immobilization decrease CatL expression.	Increased bowel movements and red stool color. High doses may cause stomach pain.	Shibaguchi et al., 2016
7	Dexamethasone	Immunomodulatory drug	Inhibits CatL and CatB activities and affects ornithine decarboxylase activity in Syrian hamster embryo cells. Reduces LPS-mediated increase of CatL mRNA level and enzyme activity by 43% ($P < .001$) and 53% ($P < .05$), respectively in muscle cell during endotoxemia.	Immune suppression; fluid retention; central obesity.	Nguyen-Ba et al., 1994; Crossland et al., 2010
8	IFN-γ	Anti-inflammatory agent	Decreases CatL activity in cultured macrophages.	Fever; headache; chills; myalgia; or fatigue; rash; injection site erythema or tenderness; diarrhea and nausea; and leukopenia.	Beers et al., 2003
9	Clenbuterol	Selective 2-adrenergic agonist	Short-term treatment with Clenbuterol mitigates denervation-induced atrophy of the soleus muscle by stimulating protein synthesis, and down-regulation of CatL and ubiquitin ligase activities.	Nervousness; thyrotoxicosis; tachycardia; subaortic stenosis; high blood pressure.	Goncalves et al., 2012.
10	Heparin	Anticoagulant	Acts as a cofactor in serpin cross-class inhibition of cysteine proteases.	Hemorrhage; heparin-induced thrombocytopenia.	Higgins et al., 2010

* Drugs are sorted by the order of categories: antimicrobial drugs, antimalarial drugs, immunomodulatory drugs, and others as discussed in the text.

phosphate) in COVID-19 patients (Fox, 1993; Yao et al., 2020). A further mechanism of chloroquine relative to the CatL function discussed here is its activity in raising endosomal pH (Al-Bari, 2017), thereby non-selectively inactivating all endosomal proteases including CatL whose optimal activity requires an acidic environment (Libby, Bursztajn, & Goldberg, 1980). By raising the endosomal pH, chloroquine blocks the proteolysis of virus surface spike protein S1 subunit in the endosomes and reduces the viral genetic material release. However, increased endosomal pH could interfere with all endosomal proteases, including CatS and CatL, which process and present viral antigens to activate T cells and to enhance antibody production (Fig. 4, left side). It is presently unknown whether COVID-19 patients showed altered cytotoxic T-cell activity or anti-viral antibody titers after chloroquine treatment *versus* other therapies. We hypothesize here that chloroquine and congeners could impair adaptive immune responses. Although detailed mechanisms remain unknown, numerous cases of COVID-19 patients have become viral positive and relapsed after hospital discharge (Shi et al., 2020). This scenario could result from defective adaptive immunity. In this regard, CatL inhibitors, or the proposed protease inhibitor cocktail therapy, could have advantages over chloroquine. Patients or cells treated with CatL specific inhibitors, with or without camostat mesylate or nafamostat mesylate, unlike those treated with chloroquine, should not display a reduction in the favorable activity of the other endosomal proteases. These proteases, including CatS (Beers et al., 2005), degrade viral proteins, generate antigenic viral peptides, and assist MHC class-1 and MHC class-II antigenic peptide loading and presentation in the endosomes (Fig. 4, right side). Therefore, host cell MHC-I- and MHC-II-mediated antigen presentation and $CD8^+$ and $CD4^+$ T-cell activations would remain unaffected. Earlier studies showed that CatL or CatS can mediate invariant chain proteolysis and antigen presentation in thymic and intestinal epithelial cells, while CatS cleaves invariant chain involved in antigen presentation by professional antigen presenting cells (APCs) (Beers et al., 2005; Nakagawa et al., 1998; Shi et al., 1999). Likewise, bronchial and alveolar type-2 epithelial cells constitutively express MHC class-I and MHC class-II molecules (Corbiere et al., 2011; Gereke, Jung, Buer, & Bruder, 2009; Wosen, Mukhopadhyay, Macaubas, & Mellins, 2018). Maintenance of endosomal protease activity in lung epithelial cells to maintain or enhance cytotoxic T-cell activity and antibody production may prove salutary during and after coronavirus infection. In contrast, chloroquine and its derivatives will raise the endosomal pH and inactivate all endosomal proteases, including CatL and CatS. Chloroquine's potential for limiting coronavirus infection might come at the cost of impairing the adaptive immune responses, a mechanism by which these drugs may benefit autoimmune

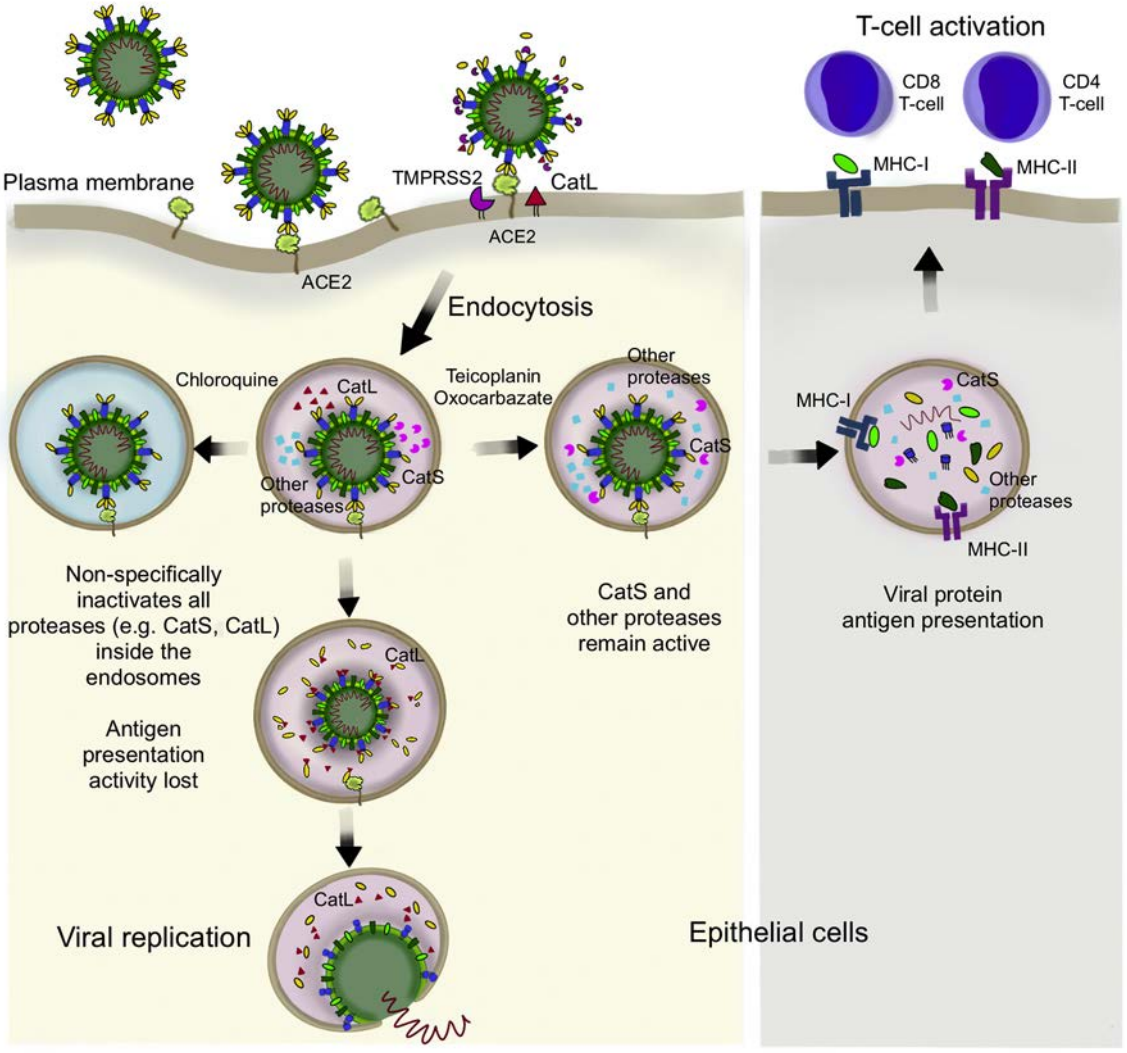

Fig. 4. Advantage of protease inhibitor cocktail therapy over chloroquine in the treatment of patients with coronavirus infection –Epithelial cells. Both serine protease TMRPSS2 and CatL appear to be involved on the epithelial cell plasma membrane S1 subunit cleavage to assist coronavirus endocytosis. Chloroquine and its analogs raise endosome pH and non-selectively inactivate all endosomal proteases including CatL and CatS, thereby blocking S1 subunit proteolysis and CatL and CatS activities in antigen presentation (left side). In contrast, CatL-specific inhibition (*e.g.* teicoplanin and oxocarbazate) selectively blocks S1 subunit proteolysis, leaving other endosomal proteases active for their essential roles in antigen processing and presentation. Remaining endosome proteases, including CatS, generate antigenic peptides and assist MHC-I- and MHC-II-mediated antigen presentation and $CD8^+$ and $CD4^+$ T-cell activation (right side).

diseases such as lupus erythematosus (Figueroa-Parra, Gamboa-Alonso, De-Leon-Ibarra, & Galarza-Delgado, 2019). Although lung epithelial cells are not the major cell type responsible for T-cell activation and antibody production, chloroquine and congeners could mute the antigen processing and presentation functions in the endosome (Fig. 4, left side).

3. Chloroquine nonspecifically impairs APC functions.

Professional APCs, such as B cells and dendritic cells, initiate specific cytotoxic T-cell activation and antibody production. Prior studies showed that coronavirus infection reduces the expression of MHC class-I and MHC class-II molecules and associated molecules (Josset et al., 2013; Menachery et al., 2018) thereby reducing anti-viral cytotoxic $CD8^+$ T-cell (Liu et al., 2010) and $CD4^+$ T-cell activation (Yang et al., 2009). Chloroquine-mediated endosomal pH increase will result in APC loss-of-function because of endosomal protease inactivation (Fig. 5, left). In contrast, CatL inhibitors (*e.g.* teicoplanin and oxocarbazate) will not affect the activities of CatS and other endosomal proteases necessary for viral antigen processing and presentation in APCs. Therefore, these agents, unlike chloroquine, will not affect the APC functions in $CD4^+$ and $CD8^+$ T-cell activation and anti-viral antibody production (Fig. 5, right).

As illustrated in Figs. 4 and 5, CatL inhibition, either with its selective inhibitor or in a protease cocktail with the serine protease inhibitor camostat mesylate or nafamostat mesylate proposed here, might protect COVID-19 patients from coronavirus infection without affecting the adaptive immunity. Three consequences of CatL inhibition could provide benefit in COVID-19. First, CatL inhibition blocks coronavirus surface spike protein S1 subunit cleavage on host cell surface, as a mechanism to block coronavirus initial infection. Second, CatL inhibition blocks virus membrane fusion in host cell endosomes, as a mechanism to block coronavirus replication. Third, CatL-selective inhibition leaves other endosome proteases active for coronavirus protein processing and antigen presentation in epithelial cells, APCs, and other non-professional APCs for T-cell activation and anti-viral antibody production.

2. Conclusion

The worldwide and severe COVID-19 pandemic calls for treatments before and even after vaccines become available. This article highlights the importance of CatL in coronavirus infection, particularly SARS-CoV-2, and proposes a hypothesis for treament of COVID-19 by

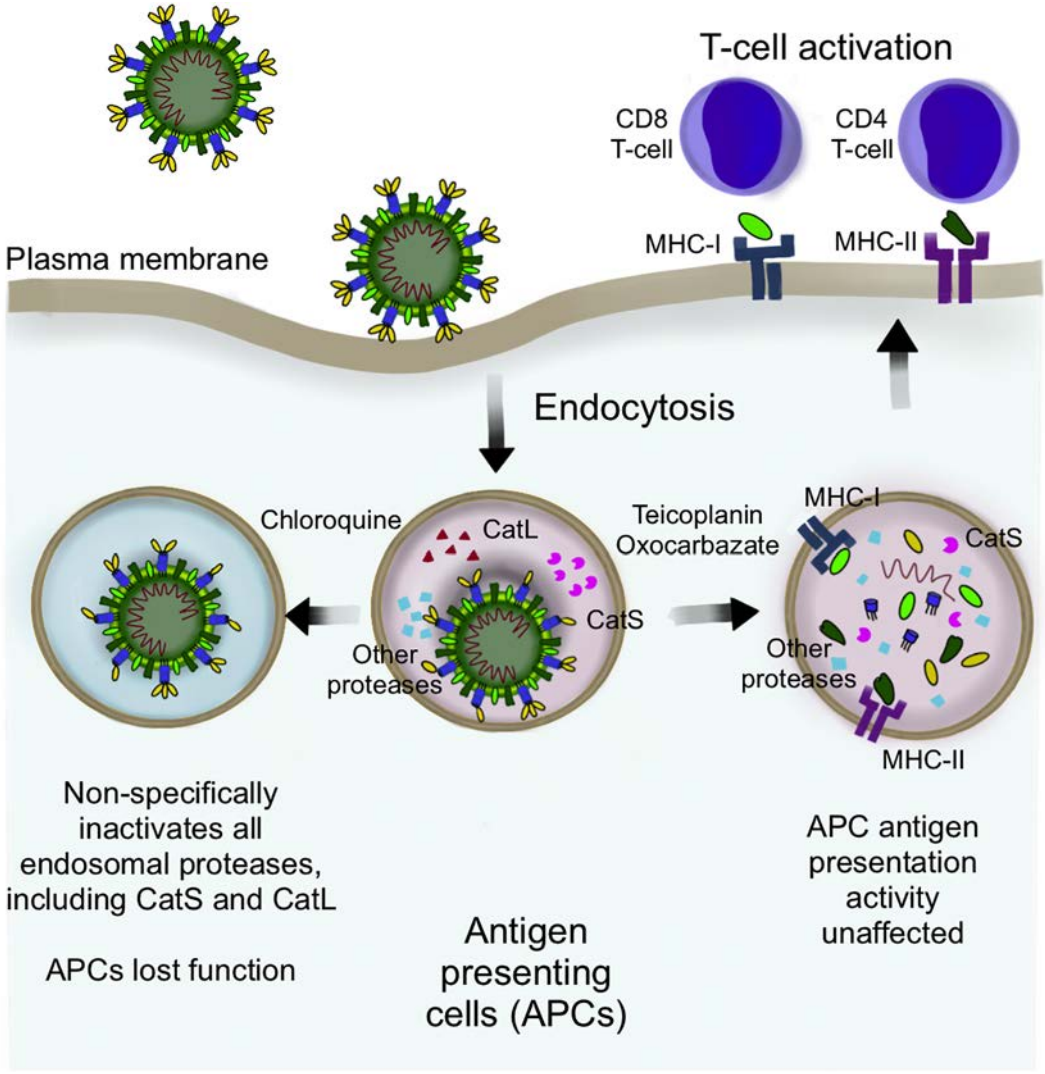

Fig. 5. Advantage of protease inhibitor cocktail therapy over chloroquine in the treatment of patients with coronavirus infection –professional APCs. Coronavirus endocytosis into the endosomes may be proteolytically processed by CatS, CatL, and other proteases. Chloroquine adversely blocks all these protease activities and APCs fail to process and present viral antigens (left side). In contrast, CatL-selective inhibitors (*e.g.* teicoplanin and oxocarbazate) do not affect other proteases, including CatS. CatS remains active to assist MHC-I and MHC-II-mediated antigen presentation and T-cell activation (right side).

targeting CatL activity. This publication also aims to bring to light existing CatL inhibitor compounds, particularly FDA-approved drugs, that may potentiate treatments for the current COVID-19 pandemic. These FDA-approved CatL inhibitory drugs provide a COVID-19 antiviral therapy that may be appropriate for patients depending on their current medical conditions, especially for those newly exposed to the virus or who test positive for the virus but are asymptomatic, when the early infection may be more clinically manageable. Therapeutic applications of CatL inhibitors, including some currently FDA-approved, employed alone or more preferably together with the serine protease inhibitor camostat mesylate or nafamostat mesylate, may emerge from both basic and clinical studies as proving value in COVID-19 treatment. As camostat mesylate and nafamostat mesylate have broad use among chronic pancreatitis patients, and a number of drugs that can inhibit CatL already have FDA approvals, we advocate testing of this protease inhibitor cocktail approach for management of COVID-19 patients. We also point out how chloroquine and analogs may impair the human adaptive immune system ability to fight virus infection. In contrast, the proposed protease inhibitor cocktail approach may both inhibit virus infection and activate adaptive immunity. We advocate testing the combined inhibition of CatL and the serine protease TMPRSS2 as a novel treatment for COVID-19 patients.

3. Discussion

In the absence of clinical trials for our proposed dual protease inhibitor therapy, we can merely speculate regarding its efficacy in COVID-19 patients and cannot predict possible adverse effects of this approach. As discussed, CatL inhibition may impair $CD4^+$ T-cell thymic selection (Honey et al., 2002; Nakagawa et al., 1998). We recently showed that chronic use of cathepsin inhibitors may associate with tissue fibrosis (Fang, Deng, Benadjaoud, Yang, & Shi, 2020; Zhang et al., 2019). Pulmonary fibrosis during the healing of acute lung injury due to COVID19 infection could be a concern. These possible adverse effects may be avoided as CatS is the major endosomal proteases that mediates antigen presentation and antibody production (Beers et al., 2005; Shi et al., 1999) and short-term use of CatL inhibitors for acute coronavirus infection should not affect tissue fibrosis appreciably.

Camostat mesylate from bio-japan.net is relatively inexpensive (100–100 mg tablets list at $39.90). Six pills a day for chronic pancreatitis treatment costs about $2.00. The on-going COVID-19 trial (NCT04352400) uses nafamostat mesylate at about one tenth of camostat mesylate by intravenous injection, although detailed dose information is not available. Patients with post-ERCP (endoscopic retrograde cholangiopancreatography) pancreatitis use 20-50 mg per day

(Park et al., 2011) that costs only $0.73 per 500 mg according to ndrugs.com. For patients with organ infections, a maximum of 600–1000 mg teicoplanin per day intramuscular administration (Rybak, 1993) is recommended, depending on creatinine clearance. Targocid from Sanofi Aventis is currently not used in the United States, but widely in Europe, Asia, and South America. It costs about $22.00 each 400 mg for injection or oral dose based on ndrugs.com, or $44.00 per day. This article proposes urgent consideration of collaborators, physicians, COVID-19 patients, and medical centers to explore this promising possibility to fight for COVID-19 and possible mutants that may develop.

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Author contributions

All authors made substantial, direct and intellectual contribution to the work and approved it for publication.

Conflict of Interest Statements.

Dr. Peter Libby is an unpaid consultant to, or involved in clinical trials for Amgen, AstraZeneca, Esperion Therapeutics, Ionis Pharmaceuticals, Kowa Pharmaceuticals, Novartis, Pfizer, Sanofi-Regeneron, and XBiotech, Inc. Dr. Libby is a member of scientific advisory board for Amgen, Corvidia Therapeutics, DalCor Pharmaceuticals, IFM Therapeutics, Kowa Pharmaceuticals, Olatec Therapeutics, Medimmune, Novartis, and XBiotech, Inc. Dr. Libby serves on the Board of XBiotech, Inc. Dr. Libby's laboratory has received research funding in the last 2 years from Novartis. Dr. Libby has a financial interest in Xbiotech, a company developing therapeutic human antibodies. Dr. Libby's interests were reviewed and are managed by Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

All other authors disclose no conflict of interest.

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