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REVIEW

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Targeting SARS-CoV-2 viral proteases as a therapeutic strategy to treat COVID-19

Varada Anirudhan[1] | Hyun Lee[2] | Han Cheng[1] | Laura Cooper[1] | Lijun Rong[1]

[1]Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, Illinois, USA

[2]Department of Pharmaceutical Sciences, Center for Biomolecular Sciences, College of Pharmacy, Biophysics Core at Research Resources Center, University of Illinois at Chicago, Chicago, Illinois, USA

Correspondence
Laura Cooper and Lijun Rong, Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA.
Email: lcoope5@uic.edu and lijun@uic.edu

Abstract

The 21st century has witnessed three outbreaks of coronavirus (CoVs) infections caused by severe acute respiratory syndrome (SARS)-CoV, Middle East respiratory syndrome (MERS)-CoV, and SARS-CoV-2. Coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, spreads rapidly and since the discovery of the first COVID-19 infection in December 2019, has caused 1.2 million deaths worldwide and 226,777 deaths in the United States alone. The high amino acid similarity between SARS-CoV and SARS-CoV-2 viral proteins supports testing therapeutic molecules that were designed to treat SARS infections during the 2003 epidemic. In this review, we provide information on possible COVID-19 treatment strategies that act via inhibition of the two essential proteins of the virus, 3C-like protease ($3CL^{pro}$) or papain-like protease (PL^{pro}).

KEYWORDS

3 chymotrypsin-like cysteine protease, coronavirus main protease, COVID-19, papain-like cysteine protease, SARS coronavirus, SARS-CoV-2

1 | INTRODUCTION

Coronaviruses (CoVs) belong to the *Nidovirales* order of enveloped positive-sense single-stranded RNA viruses. Before 2002, there were only two known human CoV species, HCoV-229E and HCoV-OC43, with infections exhibiting symptoms similar to those of the common cold caused by rhinovirus. These two CoV were identified in 1965 and have been extensively studied for the following 20 years.[1] There are now seven known species of human CoVs (HCoVs): HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, MERS-CoV, SARS-CoV, and SARS-CoV-2 belonging to alpha- and beta-coronaviruses (Figure 1A).[2] About 30% of mild upper respiratory diseases are caused by HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1.[3,4] SARS-CoV and MERS-CoV, which first appeared in China in 2002 and in Saudi Arabia in 2012, respectively, caused severe health and economic crisis at the global level. Even though its infection rate is slow, MERS-CoV infections are still ongoing and between January 2020 and September 2020, 61 new cases were reported with 21 deaths. The mortality rate of MERS (30%) is about three times more than that of SARS (10%).

The recently emerged novel SARS-CoV-2, which is currently wreaking havoc worldwide, has infected 44 million individuals and caused 1.2 million deaths as of November 10, 2020 (https://coronavirus.jhu.edu/map.html). SARS-CoV-2 infection results in coronavirus disease 2019 (COVID-19) and the clinical manifestations include fever (88.7%), dry cough (67.8%), sore throat (13.9%), dyspnea (18.6%), fatigue (38.1%) and gastrointestinal symptoms (8.8%). SARS-CoV-2 is a close cousin of SARS-CoV, sharing an overall amino-acid sequence identity of 82%.[5] Based on this similarity it is reasonable to assume that knowledge of the molecular pathogenesis of SARS-CoV could help develop SARS-CoV-2 treatment strategies. Currently, the US FDA has approved remdesivir (inhibitor of SARS-CoV-2 RNA-dependent RNA polymerase [RdRp])

Abbreviations: $3CL^{pro}$, 3 chymotrypsin-like cysteine protease; CC_{50}, 50% cytotoxic concentration; CoV, coronavirus; COVID-19, coronavirus disease 2019; CPE, cytopathic effect; DUB, de-ubiquitinating; EC_{50}, the concentration of a drug that gives half-maximal effect; FIP, feline infectious peritonitis; FRET, fluorescence resonance energy transfer; hACE2, human angiotensin-converting enzyme 2; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HRV, human rhinovirus; IC_{50}, half maximal inhibitory concentration; IFN, interferon; ISG15, interferon-induced gene 15; MERS-CoV, Middle East respiratory syndrome coronavirus; M^{pro}, main protease; nsp, nonstructural protein; ORF, open reading frame; PL^{pro}, papain-like cysteine protease; RdRp, RNA-dependent RNA polymerase; RTC, replication transcription complex; SAR, structure–activity relationship; SARS-CoV, severe acute respiratory syndrome coronavirus; TGFβ1, tumor growth factor β1.

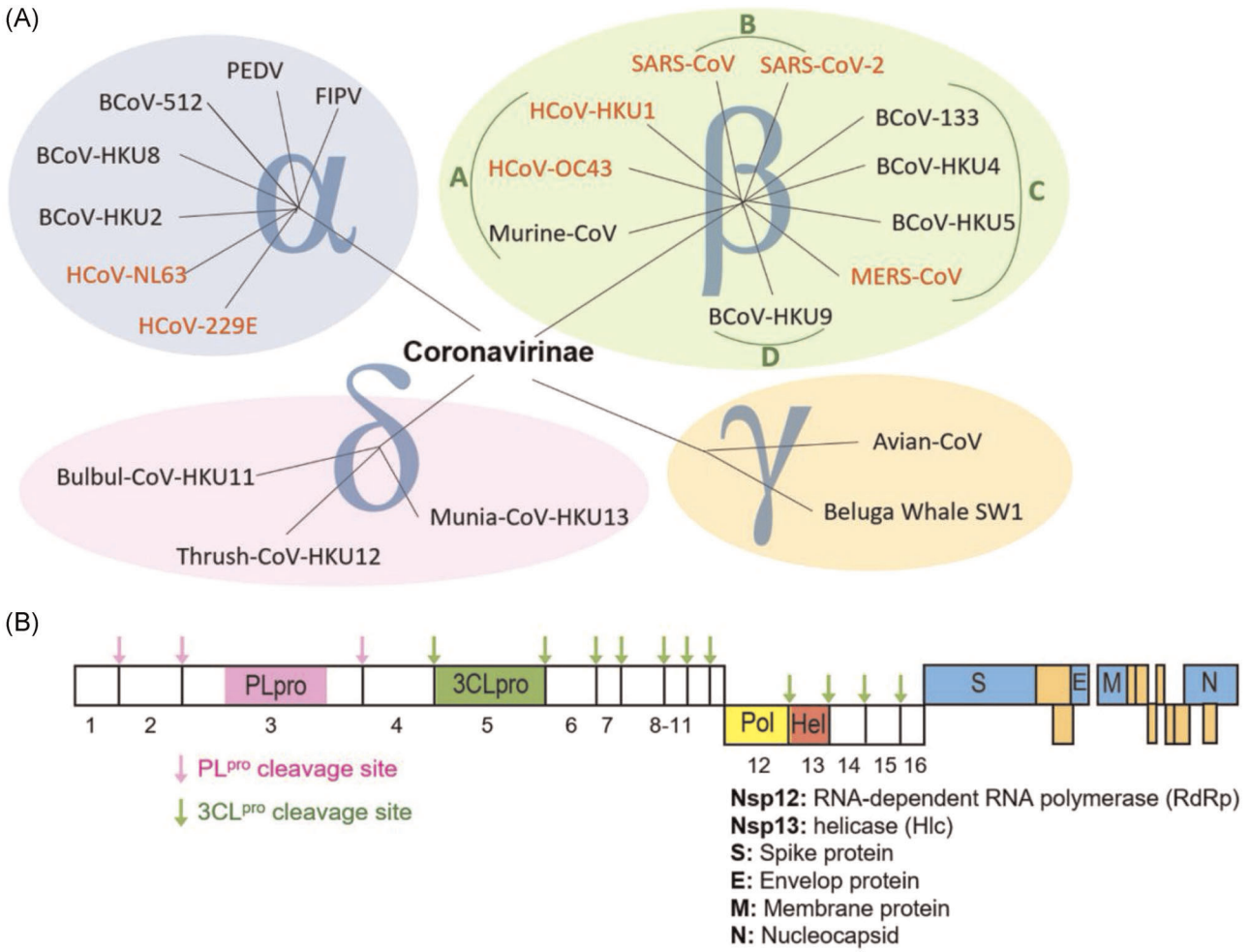


FIGURE 1 Classification of coronaviruses and polyproteins of SARS-CoV. (A) Coronavirus classification. The coronavirinae subfamily divides into four genera; alphacoronavirus, betacoronavirus, gammacoronavirus, and deltacoronavirus. Further division of the betacoronavirus into lineage subgroups is labeled in green. HCoV (human coronavirus), BCoV (bat coronavirus), PEDV (porcine epidemic diarrhea virus), FIPV (feline infectious peritonitis virus), SARS (severe acute respiratory syndrome), and MERS (middle east respiratory syndrome). Seven human coronaviruses are shown in red. (B) Schematics of the SARS-CoV polyproteins with two viral protease cleavage sites. The viral proteases PL^{pro} and $3CL^{pro}$ cleave the immature polyproteins into 16 nonstructural proteins (labeled 1–16). Pink arrows indicate SARS-CoV PL^{pro} cut sites, whereas green arrows indicate SARS-CoV $3CL^{pro}$ cleavage sites. The structural proteins include spike (S), envelope (E), membrane (M) and nucleocapsid (N)

and baricitinib plus remdesivir to treat patients with COVID-19. However, considering the large number of reported cases of COVID-19, there is an urgent call for potent SARS-CoV-2 therapeutic drugs.

A vital step in the life cycle of coronaviruses is the proteolytic processing of virally expressed polyproteins into functional units by virus-encoded proteases.[6,7] Two cysteine proteases, papain-like protease (PL^{pro}), and 3C-like protease ($3CL^{pro}$), are viral proteases encoded by the coronavirus genome; their enzymatic activities are crucial for the formation of the replication complex in the host cytoplasm. Inhibition of these viral proteases results in impaired viral replication in host cells. Thus, inhibition of SARS-CoV-2 viral proteases is a promising antiviral strategy. There is a high amino-acid percent homology that exists between the decoded SARS-CoV and SARS-CoV-2 proteases (96% for $3CL^{pro}$ and 83% for PL^{pro}). This encourages us to utilize the available information on SARS-CoV proteases to design inhibitors that potentially block activities of the SARS-CoV-2 proteases. Here, we review the characteristics of coronavirus proteases and summarize the promising inhibitory molecules targeting these proteases. We believe this information will aid in designing potential drug candidates to treat the rapidly spreading COVID-19.

2 | CORONAVIRUS GENOME ORGANIZATION

The life cycle of SARS-CoV and SARS-CoV-2 begins with their attachment to the host receptor human angiotensin-converting enzyme 2 (hACE2) via the viral surface glycoprotein known as the spike (S) protein.[8] SARS-CoV-2 S protein showed approximately 22-fold tighter binding to hACE2 than SARS-CoV S, which could be one reason why SARS-CoV-2 infection rate is much higher.[9] The Coronaviridae family

members have the largest and most complex replicating genomes of all the RNA viruses. The SARS-CoV and SARS-CoV-2 genome is about 29.8 kb long with a 5′ cap structure and 3′ polyadenylation tract.[10,11] The replicase gene (*rep*) is approximately 21 kb long and takes up around 2/3 of the 5′ region of the SARS-CoV genome. Following infection, the genomic RNA is released into the cytoplasm, and then two large polyproteins pp1a (~486 kDa) and pp1ab (~790 kDa) are synthesized from two overlapping open reading frames (ORFs) 1a and 1b that encode *rep*[10] (Figure 1B). The SARS-CoV proteases, PL^{pro} and $3CL^{pro}$, which undergo auto-catalytic cleavage post translation and aid in co-translational proteolytic processing of these two immature polyproteins to release 16 nonstructural proteins (nsps) named nsp1 through nsp16 that facilitate the formation of the multifunctional membrane-associated replication-transcription complex (RTC).[12] Furthermore, unlike other RNA viruses, SARS-CoV have an exoribonuclease domain (ExoN) in nsp14 that provides proofreading activity that protects the virus from mutagenesis.[13] The structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N) are encoded by four open reading frames that are present downstream of *rep*.

3 | SARS-COV $3CL^{pro}$: STRUCTURE AND FUNCTION

The coronavirus $3CL^{pro}$ enzyme, also known as main protease (M^{pro}), cleaves the large polyprotein pp1ab at 11 locations, releasing 13 nonstructural proteins.[14] The P1, P2, and P1′ positions of the substrate peptide are the major determinants of substrate specificity of SARS-CoV $3CL^{pro}$.[7] The P1 position has a well-conserved Glutamine residue and the P2 position has a hydrophobic core. 3CLpro recognizes and cleaves (Leu, Val, Phe, or Met)-Gln ↓ (Ser, Ala, Gly, or Asn) sequences and cleaves the polyproteins into nonstructural proteins

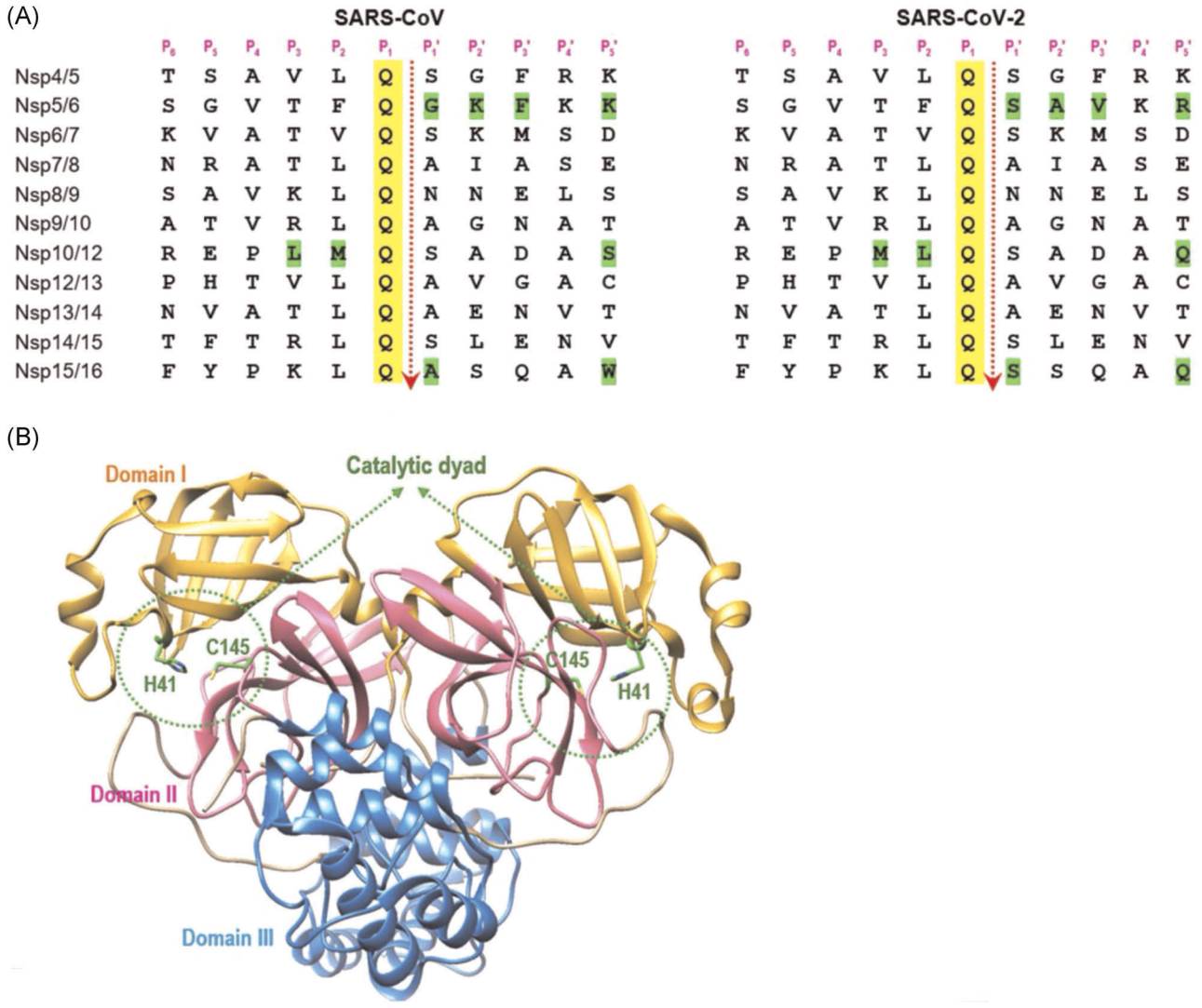


FIGURE 2 SARS-CoV 3CLpro structure and cleavage sequences. (A) Eleven cleavage sites of SARS-CoV and SARS-CoV-2 $3CL^{pro}$. Conserved residues are highlighted in yellow and highlighted in green are mismatched regions between the two $3CL^{pro}$ cleavage sites. (B) Crystal structure of the SARS-CoV $3CL^{pro}$ (PDB; 2DUC). $3CL^{pro}$ is a functional dimer. The residues 8-101 were colored in yellow (Domain I), 102–184 (Domain II) in pink, and 201–301 (Domain III) were colored in blue. The catalytic dyad (His41 and Cys145) is shown in green. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

(nsps) 4–16 (Figures 1B and 2A). While the 3CL^{pro} of other coronaviruses have leucine or isoleucine at position P2, SARS-CoV 3CL^{pro} can have either phenylalanine, valine or methionine at this position.[10,15] Sequence homology between the cleavage sites of SARS-CoV and SARS-CoV-2 3CL^{pro} is very high, and mismatching residues are highlighted in green in Figure 2A.

The crystal structure of 3CL^{pro} from SARS-CoV is similar to that from other coronaviruses and it comprises of three domains (Figure 2B).[16–18] The chymotrypsin-like structure is constructed from β-barrels contained in the domains I (residues 8-101) and II (102-184); domain III (residues 201-306) contains mostly α-helices. The active site region is located between domain I and domain II, where amino acid residues Cys145 and His41 form the catalytic dyad of SARS-CoV 3CL^{pro}.[16,19,20] Without dimerization, SARS-CoV 3CL^{pro} is inactive and the N-terminal regions of each monomer plays a crucial role in dimer formation.[21] This structural information provides a basis for prototype 3CL^{pro} inhibitors.

3.1 | Characterization of coronavirus 3CL^{pro}

The development of novel 3CL^{pro} inhibitors requires proteolytic activity assessment studies of the enzyme. A fluorescence-based assay for peptide cleavage assessment is generally used to characterize the activity of SARS-CoV 3CL^{pro}. The cleavage of the peptide is evaluated by a fluorescence resonance energy transfer (FRET) assay. The fluorescent peptide substrate comprises a fluorescent donor, a peptide, and a quenching acceptor. When the peptide is cleaved by the protease, the quenching acceptor is released which results in an increase in fluorescent signal. The most commonly used fluorescent reporter system is the Dabcyl–EDANS pair with 340 nm (excitation) and 490 nm (emission) wavelength and it typically employ the substrate molecule with the amino acid compositions: KTSAVLQSGFRKME or KNSTLQSGLRKE due to higher cleavage efficiency.[22,23] Using FRET-based peptide substrates in their cleavage assays, Grum-Tokars et al.[21] characterized the activity of SARS-CoV 3CL^{pro}. They tested the following fluorescent probes: Dabcyl-EDANS, Abz-Tyr(NO_2), Alexa488-QSY7, and Alexa594-QSY21. Upon determination of fluorescence extinction coefficient (FEC) values for the substrates, Alexa488-QSY7 was found to be the most sensitive probe. Based on the influences of assay conditions on SARS-CoV 3CL^{pro} activity in vitro, they recommended using a pH of 7.5 and less than 100 mM NaCl. They also reported drastic differences in kinetic parameters when additional amino acid residues were incorporated in SARS-CoV 3CL^{pro} and suggested the use of the enzyme without any changes to its N- or C-termini. Observed discrepancies between the enzyme kinetic parameters of SARS-CoV 3CL^{pro} reported by other research groups have also been addressed.[22–26] These variations pose a severe problem in the screening process for inhibitory drugs, and therefore a standard method of enzyme activity assessment that best mimics in vivo conditions are warranted. The use of higher wavelength fluorophore–quencher pair such as Alexa488 and Alexa594 is beneficial to avoid interference from testing compounds. However, Alexa fluorophores are expensive, and hence another 5-FAM and QXL pair (450 nm/520 nm, excitation/emission) was also developed as an effective substrate for high-throughput screening against SARS-CoV 3CL^{pro} enzymatic assays.[27] Despite advancements in the development of 3CL^{pro} enzymatic assays, they are not an alternative for in vitro or in vivo screening in live viral systems in a biosafety level 3 facility. However, they are useful as a necessary tool to screen and characterize potential inhibitors in a nonbiosafety level 3 environment.

4 | POTENTIAL THERAPEUTIC COMPOUNDS TARGETING CORONAVIRUS 3CL^{pro}

Owing to the absolute requirement of coronavirus 3CL^{pro} for viral replication, the protease has been the major focus of antiviral development. An additional factor making this enzyme more appealing as a therapeutic target is that no known human proteases share structural homology and substrate cleavage specificity with SARS-CoV 3CL^{pro}. Here, we review promising 3CL^{pro} inhibitors that have the potential for treatment of SARS-CoV-2.

4.1 | Rupintrivir

In the past, the 3C protease of the closely related human rhinovirus (HRV) has been efficaciously targeted by inhibitors to treat common cold.[28] Rupintrivir (AG7088) developed by Agouron Pharmaceuticals, Inc. is a synthetic compound that selectively and covalently inhibits the 3CL^{pro} of HRV. Rupintrivir showed potent anti-HRV activity in vitro and the drug was formulated into a nasal spray for a double-blind, placebo-controlled Phase 2 clinical trial in 1999. The drug had minimal side effects and was efficacious in reducing viral titers and symptoms such as nasal discharge.[29] It was later advanced to treat patients with acquired infections in large-scale Phase II/III trails. However, there was a lack of efficacy in natural infection studies and it was halted for further development.

Rupintrivir was tested against SARS-CoV-2 but showed little enzyme inhibitory activity (IC_{50} value of 68 ± 7 μM).[30] This is likely due to the difference in the substrate-binding sites between HRV and SARS-CoV-2. A change in the amide bond between P2 and P3 to a methyleneketone inhibits the drug's ability to bind to the 3CL^{pro} of SARS-CoV-2.[30] However, it might be possible to make modifications to the structure of rupintrivir to enhance its affinity for SARS-CoV-2 which makes it a promising lead compound for further therapeutic development.

4.2 | Ledipasvir and velpatasvir

Between the viral proteases from SARS-CoV and SARS-CoV-2, Chen et al.[31] found 100% conservation of the sequences involved in the enzymatic reaction, substrate binding and dimer formation. A virtual screen of 7173 purchasable compounds was further conducted to

identify possible SARS-CoV-2 $3CL^{pro}$ inhibitors. Two approved hepatitis C virus (HCV) drugs ledipasvir and velpatasvir were reported as suitable candidates based on their modes of action, targets, and lack of side effects (Table 1). Though the in vitro experimental data of ledipasvir and velpatasvir inhibiting SARS-CoV-2 replication via blocking $3CL^{pro}$ activity is lacking, a human clinical trial is currently under way in Egypt for the treatment of COVID-19 with sofosbuvir (a prodrug nucleotide analog inhibitor of SARS-CoV RdRp) plus ledipasvir (ClinicalTrails.gov number, NCT04530422).

4.3 | Lopinavir and ritonavir

Lopinavir and ritonavir are human immunodeficiency virus (HIV) aspartate protease inhibitors and were approved by the United States Food and Drug Administration (FDA) in 2000 for the treatment of HIV (Table 1). These two drugs are often used together because ritonavir can increase lopinavir's plasma half-life through inhibiting cytochromes P450. Lopinavir has proven inhibitory activity against MERS-CoV both in vitro (EC_{50} value of 8.0 ± 1.5 μM) and in an in vivo nonhuman primate model.[32,33] A human clinical trial for the combination of lopinavir/ritonavir and interferon β-1b to treat MERS is currently under way (ClinicalTrails.gov number, NCT02845843). Lopinavir has also been shown to block the SARS-CoV $3CL^{pro}$, but the study lacked proper randomization and control groups.[34] Lopinavir/ritonavir was used in a clinical trial to treat COVID-19 patients in China, but it was shown to be ineffective.[35]

4.4 | GC376

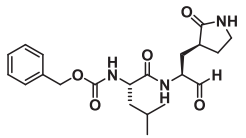
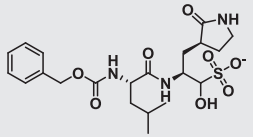
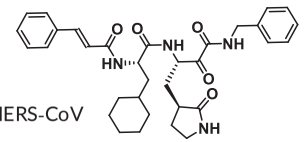
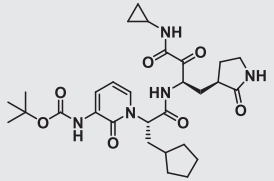
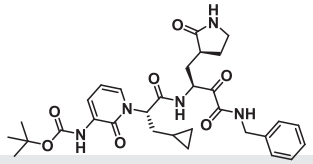
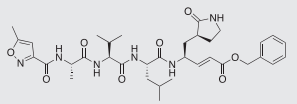
The prodrug GC376 is an approved drug for the treatment of feline infectious peritonitis (FIP) which is caused by feline coronavirus (Table 2).[36] FIP is usually fatal in cats but GC376 has shown promise in the treatment of FIP.[37] Both prodrug GC376 and its parent drug GC373 bind covalently to the catalytic Cys145 of SARS-CoV-2 $3CL^{pro}$ as shown by x-ray crystallography studies.[38] Using the FRET-based assay, it was determined that GC376 blocked proteolytic cleavage activity of MERS $3CL^{pro}$ with an IC_{50} of 1.56 ± 0.09 μM.[39] It was also demonstrated that both parent and prodrug potently blocked SARS-CoV $3CL^{pro}$ protease activity with an IC_{50} value of 0.07 ± 0.02 μM and 0.05 ± 0.01 μM, respectively. Their inhibitory activities against SARS-CoV-2 protease were slightly weaker, with IC_{50} values of 0.40 ± 0.05 μM for GC373 and 0.19 ± 0.04 μM for GC376.[38] Plaque reduction assays conducted on SARS-CoV-2 infected Vero E6 cells confirmed the antiviral potency of GC373 (EC_{50} = 1.50 ± 0.30 μM) and GC376 (EC_{50} = 0.90 ± 0.20 μM); both compounds showed no notable cytotoxicity (CC_{50} > 200 μM). Moreover, these drugs significantly reduced viral titers (3-log decrease) as indicated by virus yield reduction assays. Anivive Lifesciences is working to obtain FDA approval for GC376 as a treatment of FIP in felines. In additionally, the company is initiating two preclinical studies to further evaluate the in vivo efficacy and safety of GC376 as a therapeutic for SARS-CoV-2 in humans.

TABLE 1 Compounds that have high potential to be repurposing for treating COVID-19 by inhibiting SARS-CoV-2 $3CL^{pro}$ or PL^{pro}

S. No.	Compound	Original target organism/disease	In vitro viral inhibition against coronaviruses	Developmental stage	Clinical trials reference number
1.	Ledipasvir + Sofosbuvir	Hepatitis C virus	NA	Phase 3 clinical trial	NCT04530422
2.	Lopinavir + Ritonavir + interferon β-1b	Human immunodeficiency virus	Lopinavir EC_{50} = 8.0 ± 1.5 in Vero E6 cells	Phase 2 clinical trial	NCT02845843
3.	Disulfiram	Alcohol addiction	NA	Phase 2 clinical trial	NCT04485130
4.	Isotretinoin + Tamoxifen	Cancer	NA	Phase 2 clinical trial	NCT04389580
5.	Isotretinoin	Cancer	NA	Phase 3 clinical trial	NCT04361422

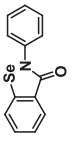
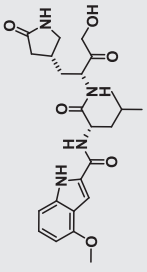
Abbreviations: COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

TABLE 2 Potential SARS-CoV-2 3CL^{pro} inhibitors

S. No.	Compound	Chemical structure	In-vitro kinetics, IC_{50} (μM)		In-vitro viral inhibition, EC_{50} (μM)		Reference(s)
			Virus	Potency	Cell line	Potency	
1.	GC373		SARS-CoV-2	0.40 ± 0.05	Vero E6	1.50 ± 0.30	72
2.	GC376		SARS-CoV	4.35 ± 0.47	Not tested		85
3.	11r		SARS-CoV	0.71 ± 0.36	Vero E6	2.10 ± 1.2	17
			MERS-CoV	Not tested	Vero E6	5.00 ± 0.4	
			SARS-CoV-2	0.18 ± 0.02	Not tested		
4.	13a		SARS-CoV-2	2.39 ± 0.63	Not tested		41
5.	13b		SARS-CoV	0.90 ± 0.29	Calu-3	1.75 ± 0.3	
			MERS-CoV	0.58 ± 0.22	Not tested		
			SARS-CoV-2	0.67 ± 0.18	Calu-3	4–5	
6.	N3		SARS-CoV-2	125	Vero E6	16.77 ± 1.7	43

(Continues)

TABLE 2 (Continued)

S. No.	Compound	Chemical structure	In-vitro kinetics, IC_{50} (μM)		In-vitro viral inhibition, EC_{50} (μM)		Reference(s)
			Virus	Potency	Cell line	Potency	
7.	Ebselen		SARS-CoV-2	0.67 ± 0.09	Vero E6	4.67 ± 0.8	
8.	PF-00835231		Not tested		$A549^{+ACE2}$	0.221	68

Abbreviations: Middle East respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

4.5 | Peptidomimetic α-ketoamides

The broad-spectrum antiviral ability of certain peptidomimetic α-ketoamides was assessed in Vero E6 cells.[40] Compound 11r was found to be a potent antiviral against SARS-CoV with an EC_{50} value of 2.10 μM and against MERS-CoV with an EC_{50} value of 5.00 μM. By modifying the chemical structure of 11r, a more stable compound 13a was designed specifically to inhibit SARS-CoV-2 $3CL^{pro}$.[41] However, 13a had lower inhibitory potency against SARS-CoV-2 $3CL^{pro}$ (IC_{50} = 2.39 ± 0.63 μM) as compared with 11r (IC_{50} = 0.18 ± 0.02 μM). Further replacing the P2 cyclohexyl moiety of 13a by cyclopropyl generated 13b with enhanced the compound's inhibitory activity against purified SARS-CoV-2 $3CL^{pro}$ (IC_{50} = 0.67 ± 0.18 μM). In addition, compound 13b inhibited SARS-CoV-2 infection in Calu3 cells with a EC_{50} value of 4–5 μM. These exciting discoveries warrant further in vivo assessment of compound 13b to study their SARS-CoV and SARS-CoV-2 inhibitory potency, before which its specificity for the respective $3CL^{pro}$ enzymes must also be studied.

4.6 | N3 and Ebselen

A Michael acceptor inhibitor named N3 was designed using computer-aided drug design to target SARS- and MERS-CoV $3CL^{pro}$.[42] N3 could also bind SARS-CoV-2 $3CL^{pro}$ from molecular docking analysis.[43] It was further demonstrated to be an irreversible inhibitor of the protease. In addition, the crystal structure of compound N3 complexed with SARS-CoV-2 $3CL^{pro}$ was solved and N3 was shown to bind to the substrate-binding region which is located between domains I and II.[43] In the HTS of a library of around 10,000 compounds, six compounds presented themselves as possible selective SARS-CoV-2 $3CL^{pro}$ inhibitors: disulfiram, carmofur, ebselen, shikonin, tideglusib, and PX-12.[43] It should be noted that a portion of these hits are promiscuous scaffolds due to the presence of sulfhydryl groups and thus making them not a promising drug lead. However, ebselen covalently bound to $3CL^{pro}$ but only partially modified Cys145 of the catalytic dyad; therefore it might be a noncovalent inhibitor and a more promising drug lead. Furthermore, ebselen was the strongest inhibitor and had an IC_{50} value of 0.67 μM. A cell-based infection assay using Vero E6 cells demonstrated ebselen and N3 to be potent antivirals with EC_{50} values of 4.67 and 16.77 μM, respectively. The assurance of low toxicity and safety has been provided for ebselen from previous animal studies and clinical trials.[44,45] Studies directed towards further elucidation and optimization of the antiviral potentials of ebselen, N3 and related compounds would be beneficial in the process of therapeutic development to combat the highly infectious COVID-19 disease.

4.7 | 4.7 PF-00835231

A previous identified ketone-based SARS-CoV $3CL^{pro}$ inhibitor, Pfizer compound PF-00835231, was also demonstrated as a potent inhibitor of SARS-CoV-2 $3CL^{pro}$.[46] An x-ray crystal structure of the compound PF-00835231 in complex with SARS-CoV-2 $3CL^{pro}$

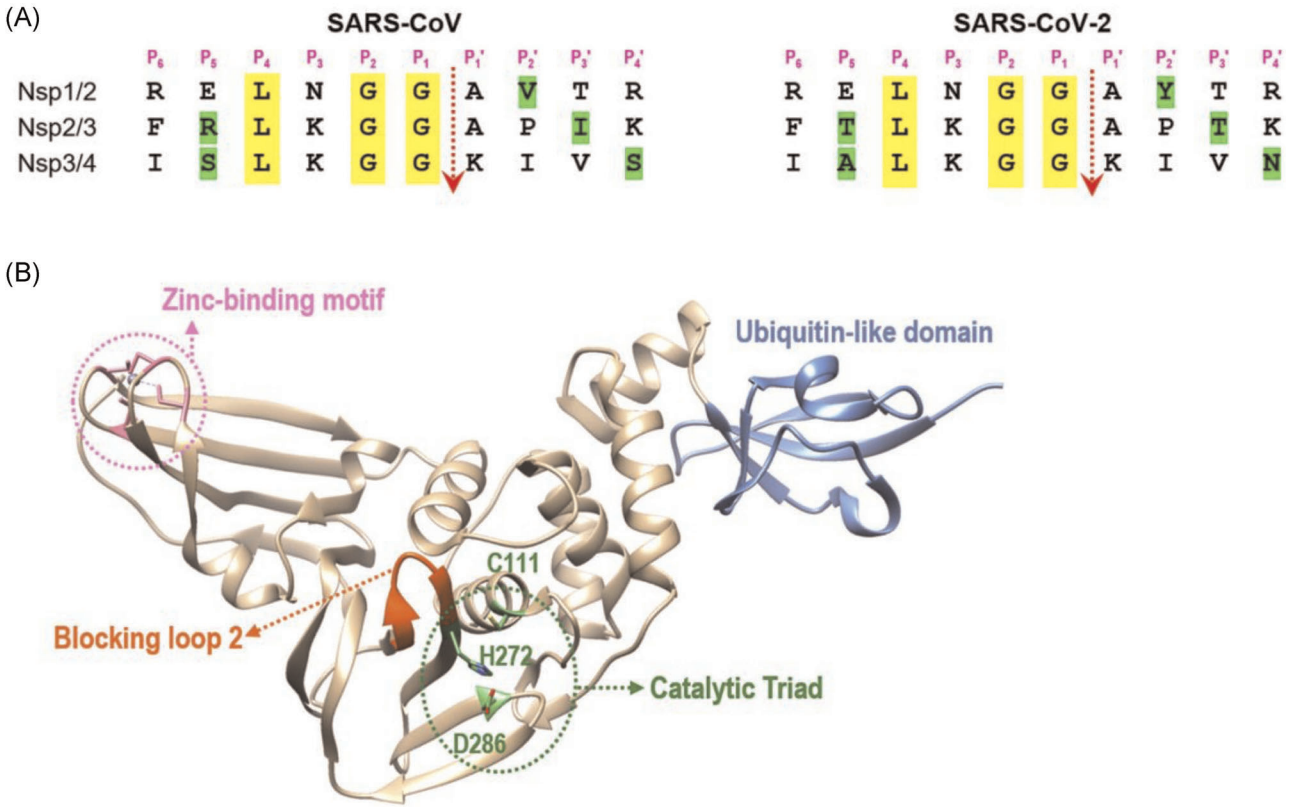
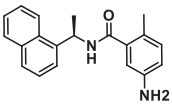
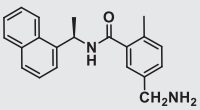
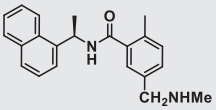
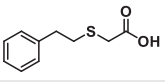
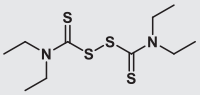


FIGURE 3 Cleavage sites and crystal structure of the SARS-CoV- PL^{pro}. (A) Three cleavage sites of PL^{pro} protease from SARS-CoV and SARS-CoV-2. Conserved residues are highlighted in yellow and highlighted in green are mismatched regions between the two PL^{pro} cleavage sites. (B) Crystal structure of SARS-CoV-2 PL^{pro} (PDB; 6WX4). The ubiquitin-like domain and Zinc-binding motif are highlighted in blue and pink, respectively. A catalytic triad is shown in the green circle and blocking loop 2 residues are in orange. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

TABLE 3 Potential SARS-CoV-2 PL^{pro} inhibitors

S. No.	Compound	Chemical structure	In-vitro kinetics, IC_{50} (μM)		In-vitro viral inhibition, EC_{50} (μM)		Reference(s)
			Virus	Potency	Cell line	Potency	
1.	GRL0617		SARS-CoV	0.6 ± 0.1	Vero E6	14.5 ± 0.8	12
			MERS-CoV	NA	Not tested		67
			SARS-CoV-2	1.5 ± 0.08			
2.	Compound 2		SARS-CoV	0.46 ± 0.03	Vero E6	6.0 ± 0.1	13
3.	Compound 49		SARS-CoV	1.3 ± 0.1	Vero E6	5.2 ± 0.3	
4.	NSC158362		Not tested		Vero E6 cells	<1	62
5.	Disulfiram		SARS-CoV	14.2 ± 0.5	Not tested		20
			MERS-CoV	22.7 ± 0.5			

Abbreviation: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

indicates that the drug binds to the enzyme via a covalent linkage with the catalytic cysteine residue (PDB:6XHM).[46]

The inhibitory potential of PF-00835231 has been confirmed in SARS-CoV-2-infected A549^{+ACE2} cells (A549 cells are inherently impermeable to SARS-CoV-2, therefore A549 cells expressing ACE2 receptor exogenously was used).[47] PF-00835231 was further evaluated with the two major currently circulating clades of SARS-CoV-2, clade A (the Wuhan basal clade) and clade B (the spike protein D614G clade) and exhibited stronger potency than remdesivir, the only drug approved by the FDA so far to treat COVID-19.[48] The reported EC_{50} values for PF-00835231 are 0.22 μM at 24 h and 0.16 μM at 48 h in clade A SARS-CoV-2-infected A549^{+ACE2} cells. The EC_{50} values of remdesivir in clade A SARS-CoV-2 infected A549^{+ACE2} cells was 0.44 μM at 24 h and 0.24 μM at 48 h. In clade B SARS-CoV-2 infected A549^{+ACE2} cells, the EC_{50} values were 0.18 μM and 0.28 μM for PF-00835231 and remdesivir, respectively. No significant cytotoxicity was observed for either compound (CC_{50} > 10 μM). PF-00835231 also exhibited strong antiviral activity in Vero E6 cells against SARS-CoV-2 infection with an EC_{50} value of 0.23 μM.[49] This assay was performed in the presence of an inhibitor of the efflux transporter P-glycoprotein (P-gp), since PF-00835231 acts as a substrate for this efflux pump.[46] In addition, PF-00835231 displayed strong antiviral infection against clade A SARS-CoV-2 in a physiologically relevant model, human airway epithelial cultures.[47] Furthermore, PF-00835231 was shown to exhibit additive/synergistic effect in combination with remdesivir in SARS-CoV-2 infected-HeLa-ACE2 cells.[49] This is conceivable since the two drugs target different steps in the life cycle of SARS-CoV-2.

Experiments conducted in vivo wherein PF-00835231 was administered intravenously (IV) to rats, dogs and monkeys indicated that the drug displayed low oral bioavailability (<2%).[49] To decrease the drug clearance and increased the bioavailability of PF-00835231 a phosphate prodrug was designed, PF-07304818, with improved ADME (absorption, distribution, metabolism, and excretion) properties and safety profile. Overall, these data encourage future clinical studies on the prodrug PF-07304818 to treat COVID-19.

5 | CORONAVIRUS PL^{pro}: STRUCTURE AND FUNCTION

In addition to 3CL^{pro}, a papain-like protease (PL^{pro}) is another attractive target for anti-SARS/MERS-CoV drug development due to its essential role in viral replication. Unlike other coronaviruses which encode two PL^{pro} paralogs, MERS-CoV, SARS-CoV, and SARS-CoV-2 produce only one copy of PL^{pro}. The 35.7 kDa-SARS-CoV PL^{pro} is part of the 213-kDa membrane-associated nonstructural protein nsp3 (Figure 1B).[50] The hydrolysis of the carboxyl side chain of the peptide backbone cleaves the SARS-CoV polyprotein pp1a at three sites ($_{177}$LNGG↓AVT$_{183}$, $_{815}$LKGG↓AP$_{821}$, and $_{2737}$LKGG↓KIV$_{2743}$; where ↓ indicates cut site), and releases three proteins nsp1, nsp2, and nsp3 which are essential for viral replication.[51] It recognizes Leu-Xxx-Gly-Gly ↓ Ala, Lys in the substrates and cleaves between Gly and Ala/Lys residues (Figure 3A). Activity profiling of SARS-CoV-2 PL^{pro} revealed that the P2 site has specificity for Gly, the P3 site can tolerate broad amino acid types and the P4 site prefers amino acids with hydrophobic side chains.[52]

The SARS-CoV PL^{pro} has four domains and three of them form distinct palm, thumb, and finger domains in addition to a ubiquitin-like *N*-terminal domain (Figure 3B).[51] A zinc ion present within a zinc-ribbon region in the finger domain was found to be a requirement for catalysis. The catalytic triad of the PL^{pro} is made up of amino acid residues Cys112-His272-Asp286. The overall sequence identity between SARS-CoV and SARS-CoV-2 PLpro proteins is 83%, and they are structurally very similar as expected. Blocking loop 2 shown in Figure 3B plays a crucial role in inhibitor binding.[53]

5.1 | Deubiquitinating and deISGylating activities of SARS-CoV and SARS-CoV-2 PL^{pro}

Studies have shown that SARS-CoV PLpro has two additional proteolytic activities, the removal of ubiquitin (Ub) and ubiquitin-like protein interferon-induced gene 15 (ISG15). SARS-CoV PL^{pro} has structural homology with the herpesvirus-associated ubiquitin-specific protease which is a cellular de-ubiquitinating (DUB) protein; thus, it is predicted to cleave a consensus sequence recognized by DUB enzymes. Barretto et al.[50] conducted in vitro studies to assess the de-ubiquitination activity of SARS-CoV PL^{pro} and demonstrated that the protease indeed possesses de-ubiquitinating potential based on its ability to hydrolyze ubiquitinated substrates. With respect to the interactions of the PL^{pro} active site with ubiquitin, biochemical and structural studies revealed that PL^{pro} interacts with ubiquitin through its palm and fingers regions and cleaves at an LXGG motif present at the P4–P1 positions of the substrate.[54,55]

Like SARS-CoV PL^{pro}, SARS-CoV-2 PL^{pro} also has deubiquitinating and deISGylating activities and the key functional differences between these two proteases were outlined by Shin et al.[11] They demonstrated that SARS-CoV-2 has host substrate preference and favorably cleaves the ubiquitin-like protein ISG15, while SARS-CoV PL^{pro} primarily cleaves ubiquitin chains.[11] In addition, the P2 position upstream of the cleavage site was shown to be the major determinant of substrate specificity. More detailed studies are needed to understand how these differences contribute to pathogenic outcomes of SARS-CoV and SARS-CoV-2 infections.

One way that SARS-CoV manipulates the host innate immune response is by the interferon (IFN) antagonist feature of its PL^{pro}. A 2006 study reported higher levels of pro-inflammatory cytokines observed in SARS-CoV infected cells.[56] Contrasting observations were made by Frieman et al.[57] who demonstrated that the SARS-CoV PL^{pro} blocked NF-κB thereby preventing IFN-mediated defense mechanisms. To address these observed discrepancies, Chen et al.[58] conducted in vitro analysis in SARS-CoV infected 293T cells and indicated that SARS-CoV PL^{pro} can interact with TRAF3, STING, and TBK1 and disrupt the STING-TRAF3-TBK1 complex which is required for IFN-β production pathway activation.

Apart from its effects on the human innate immunity, cell culture-based studies in human promonocytes provided evidence that SARS-CoV PL^{pro} stimulates tumor growth factor β1 (TGFβ1) synthesis.[59] The elevated levels of TGFβ1 has also been observed in the lungs of SARS-CoV patients and are correlated with the "pro-inflammatory storm" in the lungs.[58]

5.2 | Characterization of coronavirus PL^{pro}

A FRET-based assay involving a fluorogenic substrate peptide (similar to that described earlier for SARS-CoV $3CL^{pro}$) has been developed to assess the proteolytic activity of PL^{pro}.[50] Ubiquitin and ISG15-based fluorescence substrates were also frequently used for the PL^{pro}.[60] The sequences of the substrates were designed based on the cleavage site for SARS-CoV PL^{pro} and substrate peptides including RLRGG, RELNGG, RELNGGAP, and RELNGGAPI were used with either 7-amido-4-methyl coumarin (AMC) or Dabcyl-EDANS as fluorescent probes.[50,61]

6 | POTENTIAL THERAPEUTIC COMPOUNDS TARGETING CORONAVIRUS PL^{pro}

PL^{pro} is an attractive therapeutic target due to its essential role in viral replication and its ability to interfere with the host immune response. Inhibitory compounds with sub-micromolar activities were identified from in vitro SARS-CoV infected cell culture studies.[53,61–64] Most of these inhibitors bound to a region away from the SARS-CoV PL^{pro} catalytic site. The explanation for the lack of inhibitors targeting the active site is that they may also inhibit the host DUBs and thus results in cell-toxicity, due to the high similarity in the active site architecture between SARS-CoV PL^{pro} and host-encoded DUBs.

As previously stated, SARS-CoV-2 shares a high amino-acid sequence similarity to SARS-CoV. Therefore, previously identified SARS-CoV PL^{pro} inhibitors have a strong likelihood of inhibiting SARS-CoV-2. There have been many compounds that are reported to inhibit SARS-CoV PL^{pro} and a handful that have been validated as inhibitors of SARS-CoV-2. Here, we describe the previously identified inhibitors of SARS-CoV PL^{pro} and compare their structures, activity, and toxicity (Table 3).

6.1 | Naphthalene-based inhibitors

A fluorescence-based assay with a fluorogenic ubiquitin-like peptide substrate RLRGG-AMC was used in a high-throughput screen (HTS) and two naphthalene-based SARS-CoV PL^{pro} inhibitors 7724772 and 6577871 were identified (Table 3). Though 7724772 and 6577871 had IC_{50} values of 20.1 ± 1.1 μM and 59 μM against SARS-CoV PL^{pro} protease activity, respectively, they showed no inhibition against SARS-CoV replication.[53,63] Compound 7724772 has a stereocenter and is a racemic mix of 2-methyl-*N*-[1-(2-naphthyl)ethyl]benzamide. Each enantiomer was tested individually against PL^{pro}, and the R-enantiomer had a higher inhibitory potential with an IC_{50} value of 8.7 ± 0.7 μM. However, R-7724772 still lacked the ability to inhibit SARS-CoV replication. Further optimization on this compound led to a more potent compound designated GRL0617.[53] Kinetic studies revealed that GRL0617 is a noncovalent competitive inhibitor of SARS-CoV PL^{pro} with an IC_{50} value of 0.6 ± 0.1 μM. The antiviral activity of this compound was assessed in Vero E6 cells against SARS-CoV infection and the EC_{50} was calculated to be 14.5 ± 0.8 μM with no cytotoxicity observed at the highest concentration tested (50 μM). Since SARS-CoV PL^{pro} functions as a deubiquitinating and deISGylating (cleaves ubiquitin-like modifiers like ISG15) and there are over 50 putative deubiquitinating enzymes in humans, the selectivity of GRL0617 for SARS-CoV PL^{pro} was tested. It was observed that DUB-like enzymes such as HAUSP, USP18, UCH-L1, UCL-L3, and a PL^{pro} from HCoV-NL63 was not inhibited by GRL0617.

The x-ray crystal structure of GRL0617 in complex with SARS-CoV PL^{pro} was elucidated at a resolution of 2.5 Å which provided structural foundation for further structure–activity relationship (SAR) studies.[62] A more potent compound (Compound 2) was generated with an IC_{50} value of 0.46 μM against SARS-CoV PL^{pro} protease activity and an IC_{50} value of 12.5 μM against SARS-CoV infection in Vero E6 cells. The methylamine derivative of Compound 2, Compound 49, had less enzyme inhibitory potency against SARS-CoV PL^{pro} (IC_{50} = 1.3 μM), but gained significantly more antiviral potency in SARS-CoV infected Vero E6 cells (IC_{50} = 2.5 μM). Both Compounds 2 and 49 did not exhibit notable cytotoxicity.

The SARS-CoV PL^{pro} inhibitor 7724772 also showed inhibition against SARS-CoV-2 PL^{pro} with an IC_{50} value of 23.5 μM. Lead compound GRL0617 was less potent against SARS-CoV-2 than SARS-CoV. The compound inhibited SARS-CoV-2's PL^{pro} enzyme with an IC_{50} value of 2.4 μM and viral replication with an EC_{50} value of 21 μM.[65] In the human epithelial cells Caco-2 infected with SARS-CoV-2, treatment with GRL0617 resulted in a dose-dependent inhibition of viral replication as assessed by cytopathic effect (CPE) studies (~100% CPE inhibitory effect was observed with 100 μM of compound). The micromolar inhibitory activity of GRL0617 makes the noncovalent naphthalene-based inhibitors a good starting scaffold for further SARS-CoV-2 therapeutics development.

The structure of GRL0617 complexed with the SARS-CoV-2 PL^{pro} has been solved and revealed that the inhibitor binds in the S3-S4 pockets of the substrate cleft. Binding of the inhibitor causes the closure of the BL2 loop (Figure 3B) and narrows the substrate cleft as opposed to the endogenous ligand which enlarges it. This suggests that GRL0617 inhibits the SARS-CoV-2 PL^{pro} by preventing binding of the LXGG motif of the substrate.[60] The structural studies also suggested that a conserved amino acid reside Tyr269 is involved the inhibition of the enzymatic activity by the compound.[11] Indeed, GRL0617 lost its inhibitory activity against the mutated SARS-CoV-2 PL^{pro} in which Tyr269 was replaced by either Thr or Gly. Collectively, these studies strongly encourage further understanding of the therapeutic effects of GRL0617 class of small molecules in mitigating

COVID-19. This crystallographic structure can aide in rational drug design, leading to a new generation of naphthalene based PL^{pro} inhibitors.

6.2 | Yeast-based NSC158362 inhibitor

A yeast-based screening methodology was described by Frieman et al.[66] wherein small molecules that inhibit SARS-CoV multiplication was identified on the basis that unnatural expression of SARS-CoV PL^{pro} in *Saccharomyces cerevisiae* resulted in a much slower growth rate. Five compounds were selected from a manual screen of around 2000 compounds from the NIH Developmental Therapeutics Program (DTP) Diversity Set library. Amongst these, compound NSC158362 inhibited SARS-CoV replication (EC_{50} < 1 μM) in virus infected-Vero E6 cells and it was not cytotoxic at the highest concentration tested (100 μM). In a more physiologically relevant model of SARS disease, the compound NSC158362 considerably lowered SARS-CoV viral titers (>50-fold reduction) in infected human airway epithelial cells (HAEs). More research is needed to test the effects of NSC158362 against SARS-CoV-2 PL^{pro} and SARS-CoV-2 infection.

6.3 | Disulfiram

Another interesting FDA-approved drug, disulfiram, is capable of blocking enzymatic activities of hepatic alcohol dehydrogenase, methyltransferase, urease, and kinase (Table 1).[67,68] Because the cysteine residues of the of PL^{pro} are essential for its enzymatic activities and disulfiram can covalently bind to these residues, Lin et al.[69] hypothesized that disulfiram can block activities of coronavirus PL^{pro}. They conducted enzyme kinetic studies that provided evidence that disulfiram inhibited SARS-CoV and MERS-CoV PL^{pro} through competitive and noncompetitive mechanisms, respectively. Disulfiram inhibited DUB activities of MERS-CoV PL^{pro} and SARS-CoV PL^{pro} with IC_{50} values of 22.7 ± 0.5 μM and 14.2 ± 0.5 μM, respectively. Disulfiram also acts with FDA-approved drugs 6-thioguanine and/or mycophenolic acid synergically to inhibit MERS-CoV PL^{pro}. In addition to its antiviral activity, disulfiram's favorable safety profile and prior FDA approval make it a good example of repurposing a previously approved drug for the treatment of COVID-19. As of September 1, 2020, Disulfiram is in Phase 2 clinical trials for the treatment of COVID-19 at the University of California San Francisco. Patients who are symptomatic and COVID-19 PCR positive will be enrolled in the study and will receive 200 mg/day of disulfiram for 3 consecutive days. Patients will be monitored for SARS-CoV-2 viral load and biomarkers of inflammation (ClinicalTrails.gov number, NCT04485130).

6.4 | Isotretinoin

In a structure-based computational screen of small molecules, an antitumor drug Isotretinoin was identified as a potential SARS-CoV-2 PL^{pro} inhibitor based on the predicted enzyme binding affinity (Table 1).[70] Isotretinoin is a vitamin A derivative which was demonstrated to exhibit strong ACE2 downregulation potential.[71] It is currently in Phase 2 clinical trials to test its COVID-19 treatment potency in combination with tamoxifen (breast cancer drug) (Clinicaltrials.gov number, NCT04389580). Assessment of its efficacy to treat COVID-19 as a single treatment option is currently undergoing Phase 3 clinical trials (Clinicaltrials.gov number, NCT04361422).

7 | CONCLUSION

The current pandemic caused by SARS-CoV-2 resulting in COVID-19 urgently requires reliable potent therapeutic strategies with minimal side effects to fight it. The high sequence homology between the crucial viral proteases of SARS-CoV and SARS-CoV-2 suggests that the previously identified SARS-CoV protease inhibitors can also block SARS-CoV-2 protease activities. Here, we have provided a review of the coronavirus PL^{pro} and $3CL^{pro}$ inhibitors. Of note, GRL0617, compounds 11r and 13b, PF-00835231, and GC376 have been demonstrated to exhibit highly potent antiviral activities. Further testing these compounds in in vivo SARS-CoV-2 infection models is urgently needed for evaluating their potential as candidate drugs to treat the COVID-19 disease. Promising therapeutic drugs, sofosbuvir-ledipasvir and disulfiram are presently undergoing clinical trials to assess their safety and efficacy for treating COVID-19. It is also hopeful that a feline coronavirus treating drug, GC376, will soon enter human clinical trials to determine if it can potently and safely treat human SARS-CoV-2 infections.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Drafting of the manuscript: Varada Anirudhan and Laura Cooper. *Critical revisions of the manuscript*: Varada Anirudhan, Han Cheng, Hyun Lee, Laura Cooper, and Lijun Rong. *Supervision*: Lijun Rong.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

ORCID

Hyun Lee https://orcid.org/0000-0003-2570-8120
Laura Cooper https://orcid.org/0000-0002-6322-1260

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