

Targeting novel structural and functional features of coronavirus protease nsp5 (3CL^{pro}, M^{pro}) in the age of COVID-19

Molly K. Roe¹, Nathan A. Junod¹, Audrey R. Young¹, Dia C. Beachboard^{2,*} and Christopher C. Stobart^{1,*}

Abstract

Coronavirus protease nsp5 (M^{pro}, 3CL^{pro}) remains a primary target for coronavirus therapeutics due to its indispensable and conserved role in the proteolytic processing of the viral replicase polyproteins. In this review, we discuss the diversity of known coronaviruses, the role of nsp5 in coronavirus biology, and the structure and function of this protease across the diversity of known coronaviruses, and evaluate past and present efforts to develop inhibitors to the nsp5 protease with a particular emphasis on new and mostly unexplored potential targets of inhibition. With the recent emergence of pandemic SARS-CoV-2, this review provides novel and potentially innovative strategies and directions to develop effective therapeutics against the coronavirus protease nsp5.

HUMAN CORONAVIRUSES, DISEASE AND THE POTENTIAL FOR FUTURE EMERGING CORONAVIRUSES

Coronaviruses are enveloped, positive-strand RNA viruses responsible for a wide range of diseases in a diverse range of animal hosts. To date, seven human coronaviruses (HCoVs) have been identified to cause respiratory diseases of varying severities: HCoV-OC43, HCoV-229E, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV and SARS-CoV-2. Among these seven HCoVs, four (HCoV-OC43, HCoV-229E, HCoV-NL63 and HCoV-HKU1) are common co-circulating, seasonal coronaviruses that cause mild respiratory tract infections generally associated with cases of the common cold [1–4]. While these viruses are capable of more severe illness in more sensitive and susceptible populations, such as those that are immunocompromised, young and elderly, limited attention or support was given to the development of therapeutics or vaccines for coronaviruses until the emergence of the first of three novel pathogens of more significant disease, SARS-CoV, in 2002 [2, 5].

SARS-CoV emerged in November 2002 in Guangdong Province, China, and ultimately spread to 29 countries, infecting

over 8000 individuals, in a 2-year span [6, 7]. Severe acute respiratory syndrome (SARS) is characterized by flu-like symptoms including a high fever, myalgia, and in advanced forms of the disease commonly dyspnoea and pneumonia [8]. In contrast to the low mortality rates associated with the common cold-associated coronaviruses, SARS-CoV was the first of three HCoVs that illustrated the emerging potential for significant disease with a case fatality rate of approximately 10% [9]. Approximately a decade after the SARS-CoV outbreak, a second emerging severe coronavirus pathogen, MERS-CoV, emerged in 2012 in Saudi Arabia [10]. Like SARS-CoV, this pathogen was capable of significant lower respiratory disease with flu-like symptoms leading to dyspnoea, pneumonia and acute respiratory distress syndrome (ARDS) [11, 12]. However, MERS-CoV was associated with a far greater potential of severe disease with a case fatality rate of approximately 35–40% [12]. While both SARS-CoV and MERS-CoV have highlighted the potential for significant disease and mortality, public health efforts, barriers to human-to-human transmission and limited asymptomatic spread all hindered their pandemic potentials [13–15].

In December 2019, cases of viral pneumonia of an unknown cause first appeared in Wuhan, Hubei Province, China

Received 27 August 2020; Accepted 08 January 2021; Published 28 January 2021

Author affiliations: ¹Department of Biological Sciences, Butler University, Indianapolis, IN, USA; ²Department of Biology, DeSales University, Center Valley, PA, USA.

***Correspondence:** Dia C. Beachboard, dia.beachboard@desales.edu; Christopher C. Stobart, cstobart@butler.edu

Keywords: Coronavirus; nsp5; 3CL^{pro}; inhibitors; protease.

Abbreviations: ARDS, acute respiratory distress syndrome; ERGIC, Endoplasmic reticulum-Golgi intermediate compartment; FIPV, feline infectious peritonitis virus; HCoV, human coronavirus; HIV, human immunodeficiency virus; IDL, interdomain loop; MHV, mouse hepatitis virus; NEMO, nuclear factor- κ B essential modulator; nsp, non-structural protein; PDCoV, porcine deltacoronavirus; PEDV, porcine epidemic diarrhoea virus; PLP, papain-like protease; SAR, structure–activity relationship; SARS, severe acute respiratory syndrome.

001558 © 2021 The Authors



This is an open-access article distributed under the terms of the Creative Commons Attribution License. The Microbiology Society waived the open access fees for this article.

[16, 17]. Quickly recognized by the World Health Organization (WHO) as a 'public-health emergency of international concern' in January 2020 and later classified as a true pandemic, SARS-CoV-2 has resulted in over 67 million confirmed cases and 1.5 million deaths worldwide (as of December 2020) [18]. Studies of transmissibility of SARS-CoV-2 have indicated a greater R_0 than SARS-CoV and MERS-CoV, suggesting a greater rate of transmissibility and infectivity for SARS-CoV-2 compared to either virus [19–22]. SARS-CoV-2 spreads mostly through respiratory droplets and direct contact with asymptomatic or symptomatic infected persons [20–23]. Although all three emerging infectious diseases share fever, shortness of breath and severe pneumonia as clinical manifestations, SARS-CoV-2 disease (named COVID-19) can specifically cause systemic inflammation which can develop further into acute cardiac injuries, sepsis, abnormal organ functions and heart failure [19, 23, 24]. Other distinctive clinical features of SARS-CoV-2 include sore throat, hypoxaemia, dyspnoea, sneezing and diarrhoea [22, 23]. Unlike SARS-CoV and MERS-CoV, SARS-CoV-2 has the potential to continue co-circulating throughout the world with the four common cold-associated HCoVs because of its lower case fatality rate and greater transmissibility [25].

Research suggests that all three emergent betacoronaviruses are zoonotic and originated within different bat species [26–28]. Due to infrequent bat–human interactions, the intermediate hosts responsible for SARS-CoV, MERS-CoV and SARS-CoV-2 acquiring the appropriate mutations to infect humans were probably palm civets, dromedary camels and potentially Malayan pangolins, respectively [1, 15, 27]. Even though SARS-CoV-2 shares a considerable amount of nucleotide sequence with MERS-CoV (51.8%) and SARS-CoV (79.0%), it is most similar (with greater than 95% identity) to coronaviruses found in bats [20, 25, 27, 28]. There remain many more bat coronaviruses which have been identified and thus lack a human analogue, highlighting the continued potential for future emergent HCoVs.

Until recently, there remained no commercially available vaccines for coronaviruses and limited therapeutic options. Despite extensive investigation and hundreds of studies evaluating critical viral targets including the polymerase (nsp12; RdRp) and main protease (nsp5), there remains a critical need for the development of effective therapeutics to treat current and future coronavirus infections [29, 30].

Coronavirus replication and the role of nsp5 protease

Coronaviruses are enveloped viruses with 27–32 kb positive ssRNA (+ssRNA) genomes which are classified in four different genera (*Alpha*-, *Beta*-, *Gamma*- and *Deltacoronavirus*) within the order *Nidovirales* and family *Coronaviridae* [31]. During virus infection, coronaviruses employ trimeric spike (S) proteins to facilitate entry into host cells [32, 33]. The interaction of this protein with its receptor dictates species and tissue tropism. Among human coronaviruses, several different cellular fusion receptors have been identified,

including aminopeptidase N (HCoV-229E), angiotensin-converting enzyme 2 (HCoV-NL63, SARS-CoV and SARS-CoV-2), and dipeptidyl peptidase 4 (DPP4) [34–38]. Upon receptor binding, the viral and cellular membranes are fused together triggered by spike (S) activation through proteolytic cleavage by a cellular protease such as TMPRR2 or cathepsin [39]. Immediately upon entry, the virus translates its replicase gene (ORF1) which consists of two large, overlapping ORFs, ORF1a and ORF1ab (Fig. 1) [31]. Located at the end of ORF1a, a ribosome frame-shifting sequence consisting of an RNA pseudoknot causes the co-translation of two large polyprotein precursors of differing lengths, pp1a and pp1ab [31, 40, 41]. Polyprotein pp1a contains non-structural proteins (nsps) 1–11, and polyprotein pp1ab comprises the complete translated coding region of nsps 1–16 [42, 43]. Essential for virus replication is the proteolytic processing of these polyproteins by virus-encoded proteases to yield the mature and functionally active replication machinery of the virus [42].

Once proteolytically processed, the translation products of pp1a collectively modulate host cell factors and help prepare the cell for viral RNA synthesis through the formation of replication complexes, while the C-terminal translation products of pp1ab largely catalyse and/or regulate the processes of RNA replication and transcription driven by the viral RdRp (nsp12) [31, 44]. Replication complexes assemble on virus-induced membrane structures such as double-membrane vesicles and convoluted membranes driven by transmembrane nsps 3, 4 and 6 [45–48]. The active replication complex promotes the continuous and discontinuous synthesis of negative-sense RNA templates, which are subsequently used to drive formation of genomic copies and a nested set of subgenomic RNAs from the downstream ORFs encoding structural and accessory proteins, respectively [49]. Following replication of genomic and subgenomic RNA on double-membraned vesicles, structural proteins like the spike (S), envelope (E), matrix (M) and nucleocapsid (N) proteins are translated by existing positive-strand subgenomic RNAs. S, E and M become glycosylated within the Golgi before localizing to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) to be assembled into virions [50–53]. The N protein will localize with the replicase proteins at replication complexes within the cytoplasm while RNA is synthesized, where it is thought to encapsidate the newly made RNA [53, 54]. After RNA synthesis, genomic RNA and N protein move to the ERGIC and assimilate into budding virions. Additional S protein is expressed on the cell surface where it triggers cell-cell fusion between infected cells and nearby, uninfected cells [55]. Consequently, massive, multinucleated cell complexes called syncytia often form, facilitating spread of the virus while avoiding neutralization via virus-specific antibodies [31, 55].

As earlier mentioned, proteolytic processing acts as a key regulatory mechanism in the expression of the coronavirus replicase proteins, as blocking this process has been demonstrated to block viral replication entirely [42, 56–58]. Typically, coronaviruses code for two or three proteases to process the replicase polyprotein: one or two papain-like proteases

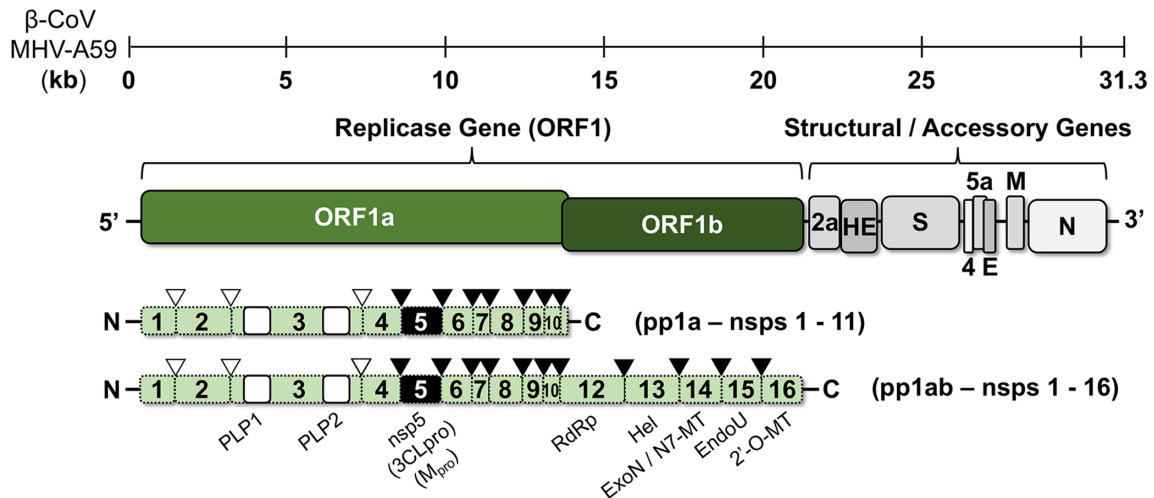


Fig. 1. Coronavirus genome organization, replicase gene expression and polyprotein processing. The 31.3 kb genome of beta-coronavirus MHV-A59 is depicted. The viral ORFs associated with replication (replicase gene; ORF1a/ORF1b) and structural and accessory genes are shown. The two variant polyproteins (pp1a and pp1ab) translated from the replicase gene are shown with the non-structural protein domains of the polyprotein labelled and the proteolytic cleavage sites marked with arrows. Three proteases mediate the proteolytic processing of the replicase polyproteins [PLP1, PLP2 and nsp5 (3CL^{pro}/M^{pro})], and the colour of the arrows (white for PLPs and black for nsp5) for each cleavage site correspond to the protease responsible for mediating its cleavage. PLP, papain-like protease; RdRp, RNA-dependent RNA polymerase; Hel, helicase; ExoN, exonuclease; N7-MT, N7-methyltransferase; EndoU, endoribonuclease; 2'-O-MT, 2'-O-methyltransferase.

(PLPs) encoded within nsp3, and one main protease, nsp5 (3CL^{pro} or M^{pro}) [31]. PLPs are responsible for cleavage events between nsp1 and the N terminus of nsp4, whereas all remaining pp1a/pp1ab cleavage events are mediated by nsp5 [42, 59–61]. In addition, both proteases have been implicated in targeting host cell targets including modulating deubiquitination, deISGylation and virus evasion of the innate immune response [60, 62].

Nsp5 protease structure and function

The coronavirus protease nsp5 (3CL^{pro} or M^{pro}) is an approximately 30 kDa, three-domain cysteine protease conserved in structure and function in all known coronaviruses and serves as the main protease for proteolytic processing of the replicase polyproteins (pp1a and pp1ab) [31, 42, 63, 64]. The name 'main protease', or M^{pro}, refers to the critical role of this protease in coronavirus gene expression and replicase processing, and its other name '3C-like protease' (3CL^{pro}) refers to the similarities between this protease and 3C proteases seen in picornaviruses, namely their similar substrate specificities and core structural homology [65]. Among coronaviruses, nsp5 proteases within the same genus generally exhibit sequence identity of greater than 80% whereas protease in different genera are far more divergent with sequence identity much closer to 50% despite high tertiary and quaternary structural conservation especially in domains 1 and 2 (Fig. 2). Unsurprisingly, the greatest degree of sequence conservation exists in and around the enzyme active site (Fig. 3). Sequence analysis of the SARS-CoV and SARS-CoV-2 proteases reveals only 12 residue differences (approximately 96% identity) spread throughout the structure of the protease, with the majority

of these residues distant from the active site (including along the distal surface of domain 1 and within domain 3), which strongly supports the prospect of developing active-site inhibitors that target both proteases.

The N-terminal domains (1 and 2) of nsp5 are structurally highly conserved and form a chymotrypsin-like fold consisting of beta-barrels which surround the substrate binding site between the two domains (Fig. 4) [63, 65, 66]. The catalytic activity of the protease is mediated by a His-Cys catalytic dyad with the Cys residue serving as a nucleophile in the enzyme-catalysed proteolytic reaction. The nsp5 protease almost exclusively orchestrates cleavage after a P1-Gln (with few exceptions) [67–70]. The P2 substrate residue is also generally well conserved with typically a Leu, although other residues can occupy this site including Met, Phe, Val or Ile [67, 69]. The P1' substrate residue typically shows much more diversity with Ser, Gly, Ala and Val residues all being found in known nsp5 cleavage sites. Far more diversity in amino acid residue usage is observed at the P2–P5 and P2'–P3' sites. Collectively, the consensus cleavage site for nsp5 across known coronaviruses is P3-PLQ-(S/G/A/V)-P1' [67]. The high specificity and consistency in cleavage site recognition among known coronaviruses has made the enzymatic active site of nsp5 the primary target for current inhibitor design efforts (Fig. 3).

While nsp5 domains 1 and 2 have been well characterized, much less is known of the role of the alpha-helical third domain of the protease. Most of the function of the helical third domain of nsp5 has been shown to direct nsp5 dimerization and help stabilize the chymotrypsin-like fold

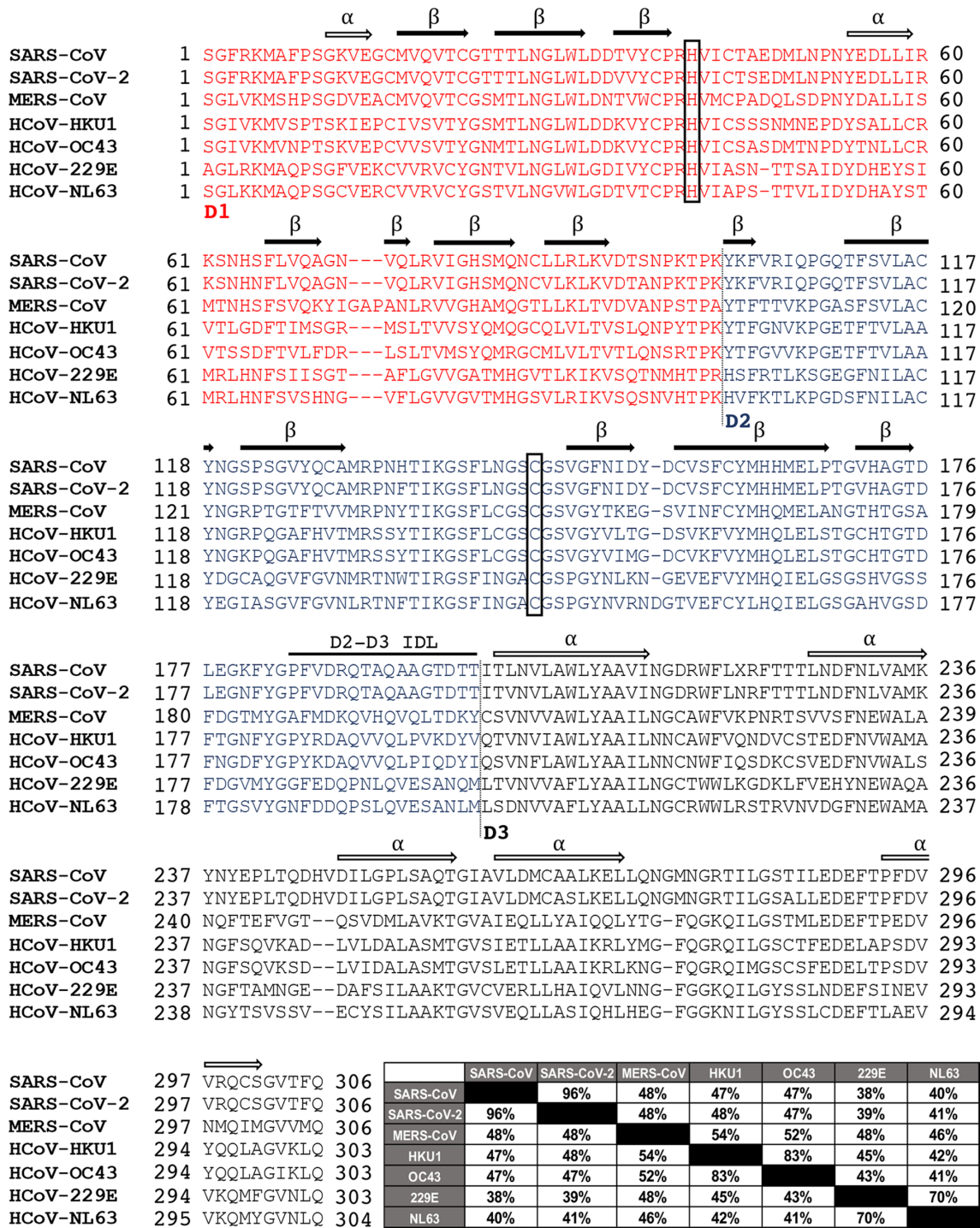


Fig. 2. Sequence alignment and conservation of the nsp5 (3CL^{pro}) protease sequences of the seven known human coronaviruses. The seven human coronavirus protease sequences were aligned and the three domains of the proteases are colour coded and labelled. Alpha helices (open arrows) and beta sheets (filled arrows) are shown and the catalytic dyad residues (His/Cys) are boxed. A conservation matrix depicting percentage identity between amino acid sequences is shown at the end of the sequence alignment.

[71, 72]. Nsp5 dimerization is essential for nsp5 function and the monomeric form of the protease is largely inactive [65, 73–75]. In the nsp5 dimer, the monomers of the protease are orientated with their active sites facing

away from one another with their N-terminal extensions (N-terminal fingers) and third domains directly interacting along a dimerization interface (Fig. 4). Studies evaluating residues important for dimerization have demonstrated that

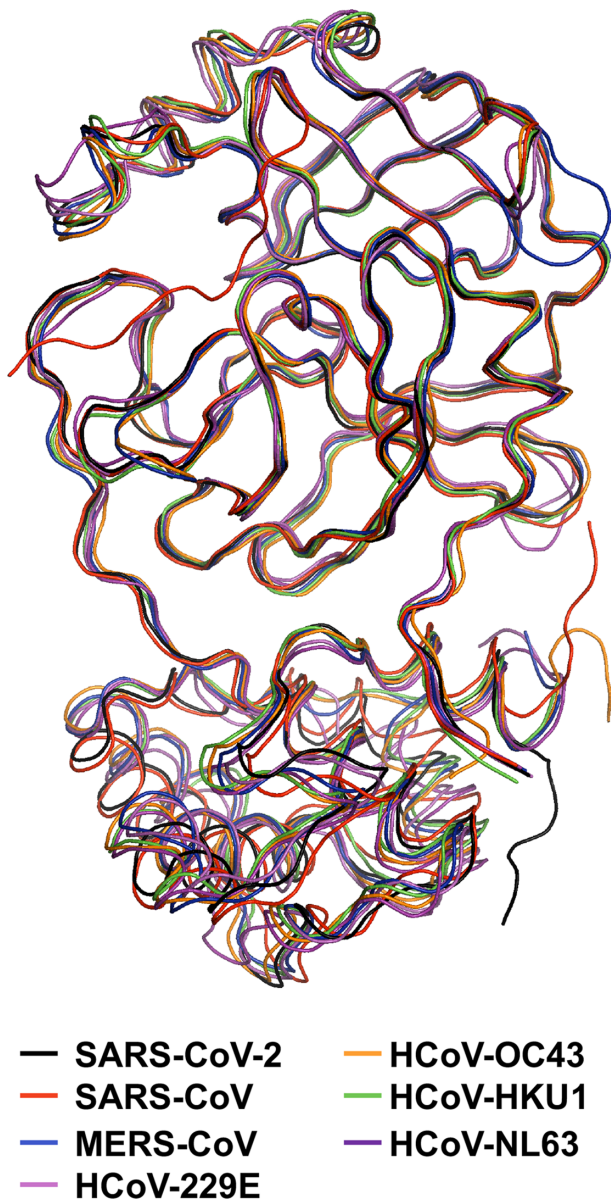


Fig. 3. Structural alignment of HCoV nsp5 (3CL^{pro}) protease crystal structures. An overlay of the monomeric crystal structures of HCoV nsp5 proteases of SARS-CoV-2 (PDB 6M2N), SARS-CoV (PDB 2Q6G), MERS-CoV (PDB 4YLU), HCoV-229E (PDB-2ZU2), HCoV-HKU1 (PDB-3D23), HCoV-NL63 (PDB-3TLO) and the modelled structure of HCoV-OC43 (PM0079872) [66, 75, 104, 139–143].

Glu166 in SARS-CoV forms critical interactions with the N-terminal finger residues of the heterologous monomer [68, 76]. Deletion of the first few residues in the N-terminal finger of SARS-CoV results in significant losses in enzymatic activity and disruption of dimerization [68, 77]. Structural and biochemical studies have demonstrated that subtle differences exist in the coordinating residues for dimerization between SARS-CoV, MERS-CoV and SARS-CoV-2 [75, 78]. In addition, it has been shown that dimerization in MERS-CoV requires ligand association, whereas

such a requirement is not present for SARS-CoV [75]. Prior to processing, nsp5 is found within a >800 kDa precursor which is processed into a 150 kDa, nsp4–10 precursor [79, 80]. nsp5 is initially responsible for coordinating its own autoproteolytic cleavage [68, 81]. It is hypothesized that two nsp5 proteases anchored to membranes by transmembrane protein nsp4 and nsp6 form a dimer and trigger cleavage *in trans* [68, 75, 81, 82]. Upon its own maturation cleavage, nsp5 is believed to target the nsp9–10 site, prior to targeting the nsp8–9 and nsp7–8 sites, respectively, for processing [83]. Once these sites are processed, the other nsp5 that nsp5 is responsible for cleaving are individually separated from the nsp7–10 site. Before the final processing of nsp5, one of the intermediate complexes, nsp7+8, conducts an important function: catalysing the cleavage of nsp12, an essential viral polymerase. Studies involving mutation of the nsp5 cleavage sites have shown that disruption of the nsp7–nsp8 and nsp8–nsp9 cleavage sites results in loss of virus viability whereas other sites such as the nsp9–nsp10 site can be tolerated with reduced replication [84]. The ordered processing by the nsp5 protease may represent a unique facet of viral replication that may be disrupted by inhibitors.

It has been suggested that nsp5 associates with numerous other components of the replication complex. Several studies have shown important intra- and intermolecular associations between the nsp5 protease and the rest of the replicase gene, with mutations both within the nsp5 domain and mutations in nsp3 and nsp10 negatively impacting nsp5 activity [80, 85, 86]. These data strongly suggest that important allosteric interactions exist between nsp5 and other members of the replicase gene. In addition, several temperature-sensitive mutations have been identified within the nsp5 proteases of mouse hepatitis virus (MHV) and HCoV-OC43 that selected for second-site compensatory mutations that were distant (>15 Å) from the initial mutation site [63, 85]. These data indicate that complex interactions which span all three domains of the protease are critical for protease structure and function. Additional studies are needed to understand their role as they may represent novel directions for proteolytic inhibition.

Therapeutic design strategies for nsp5 inhibitors

As SARS-CoV-2 continues to spread and interfere with our daily lives, a need for an effective, safe way to treat the viral infections has become much more prevalent. While the efficacy and safety of an array of vaccine candidates is currently being evaluated, it seems unlikely that worldwide distribution of available vaccines at rates needed for herd immunity will not occur for quite some time [87, 88]. In addition to the challenges of testing for safety and efficacy, vaccines target specific pathogens which make them limited in treating future problematic diseases, especially diseases caused by rapidly mutating and evolving RNA coronaviruses. Because of these concerns, the practicality of utilizing therapeutic alternatives to treat current and future viral outbreaks appears more promising [87]. Considering the heightened interactions among humans and animals, the

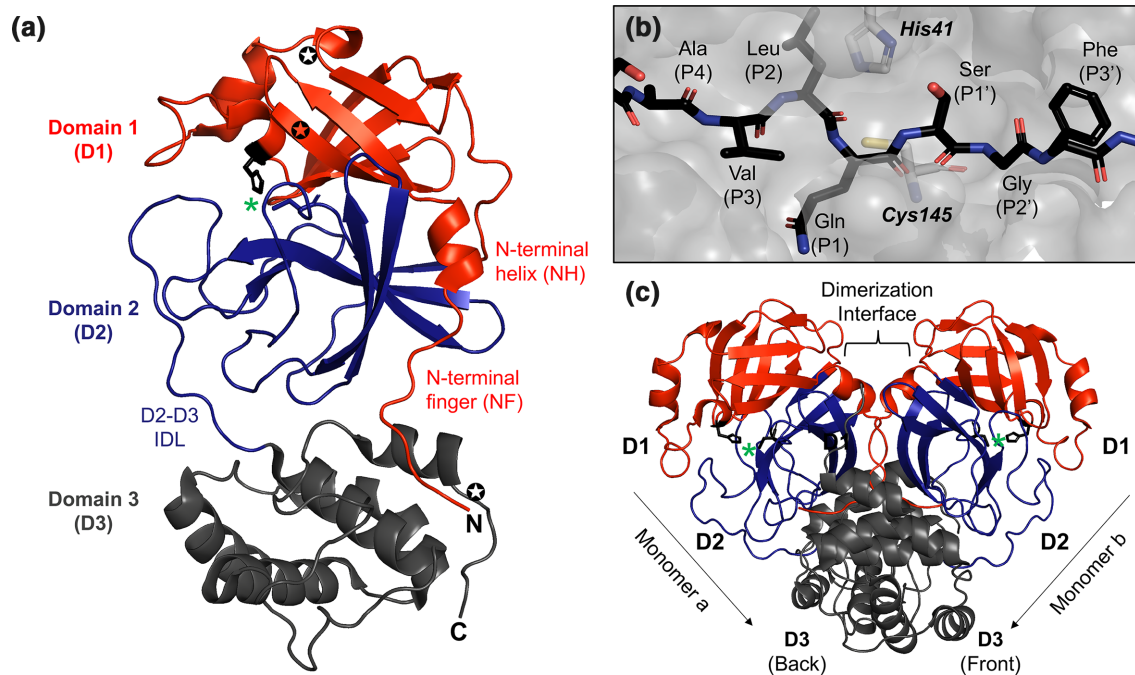


Fig. 4. Nsp5 (3CLpro) structural features of SARS-CoV-2 protease. (a) Monomeric structure of the SARS-CoV-2 nsp5 protease (PDB 6M2N) with the three domains shown. Key structural regions including the N-terminal finger (NF), N-terminal helix (NH) and domain 2–domain 3 interdomain loop (IDL) are labelled. Corresponding locations of MHV nsp5 resistance mutations identified in Deng *et al.* are labelled with stars [144]. (b) A view of the active site with a consensus cleavage site peptide bound. The catalytic dyad residues are shown. (c) SARS-CoV-2 nsp5 dimer structure with the orientation of the two dimers shown by arrows and the dimerization interface labelled. Green asterisks denote the catalytic site with black sticks shown for the catalytic dyad residues (His41 and Cys145) in both the monomeric (a) and the dimeric structures (c).

immense viral diversity characterizing coronaviruses, and frequent adaptations and mutations, targeting a conserved region among coronaviruses for therapeutic treatment might de-escalate the lingering threat of future human coronavirus outbreaks or pandemics [13].

Since the SARS-CoV outbreak of 2003–2004, there have been ongoing efforts to develop inhibitors that target nsp5. Several compounds have been designed and tested for nsp5 active-site inhibition, including esters and covalent or non-covalent peptidomimetics (summarized in Table 1) [89, 90]. The covalent modifiers (esters and peptidomimetics) act as competitive inhibitors that mimic natural peptide substrates of nsp5. The non-covalent inhibitors occupy the substrate binding site to competitively inhibit nsp5 activity. Additionally, some groups have done computational screening of known drug libraries to identify potential inhibitors [91, 92].

The first ester-based compounds to be developed were benzotriazole ester-based and irreversibly acylated the C145 within the active site [93]. Next, chloropyridine-esters were tested and found to have potent inhibitory activity against nsp5. CE-5 is one of the chloropeptidyl esters that has been extensively studied and shown to have broad activity across several CoVs. CE-5 has an EC_{50} of 8.5 μM for MHV [94], $24 \pm 0.9 \mu\text{M}$ for SARS-CoV [95] and 13.5 μM for MERS-CoV [96]. Additionally, this inhibitor was tested during BtCoV-HKU5 and

MERS-CoV infection, where it resulted in a 10–100-fold decrease in viral titres depending on the timepoint [97].

Extensive work has been done to identify peptidomimetic inhibitors of nsp5 [89]. The majority of these compounds contain Michael acceptors, chloromethyl ketone or epoxide functional groups. These functional groups form covalent, irreversible interactions with nsp5 C145. However, some of these compounds also form non-covalent interactions with the nsp5 active site. Early peptidomimetic inhibitors were designed based on AG7088, which targets the human rhinoviral 3C protease [98, 99]. While the AG7088 compound was not effective in SARS-CoV-infected cells [100], it was used to subsequently design molecules that have antiviral activity against the SARS-CoV nsp5 protease. Two groups studied the AG7088 derivative and showed potent activity on the SARS-CoV nsp5 [98, 99, 101]. Shie *et al.* identified five compounds with EC_{50} values of <20 μM , three of which were <0.2 μM . Kumar and colleagues extended the testing of 3C^{pro} inhibitors against both SARS-CoV and MERS-CoV and identified compounds with low micromolar activity and EC_{50} of 0.6–1.4 μM [102].

In addition to testing known inhibitors of 3C proteases, a structure-based design has also been used to identify nsp5 inhibitors. Using the crystal structure of MERS-CoV in complex with the antiviral compound GC376, Galasiti Kankanamalage *et al.* designed several compounds and

Table 1. Select nsp5 inhibitors

Compound	Type of compound/ source	Virus	IC ₅₀ (μM)	EC ₅₀ (μM)	References
CE-5	Chloropyridine-esters	MHV	ND	8.5	[94]
		SARS-CoV	ND	24±0.9	[95]
		MERS-CoV	ND	13.5	[96]
18a	Peptidomimetic – AG7088 derivative	SARS-CoV	10	18.86	[101]
18b	Peptidomimetic – AG7088 derivative	SARS-CoV	5	9.45	
18c	Peptidomimetic – AG7088 derivative	SARS-CoV	1	0.18	
18d	Peptidomimetic – AG7088 derivative	SARS-CoV	10	0.11	
18e	Peptidomimetic – AG7088 derivative	SARS-CoV	7	0.16	
1	Peptidomimetic – AG7088 derivative	SARS-CoV	870/45	ND	[98]
2	Peptidomimetic – AG7088 derivative	SARS-CoV	800/70	ND	
3	Peptidomimetic – AG7088 derivative	SARS-CoV	8075	ND	
4	Peptidomimetic – AG7088 derivative	SARS-CoV	10/100	ND	
5	Peptidomimetic – AG7088 derivative	SARS-CoV	15/100	ND	
16	Peptidomimetic – AG7088 derivative	SARS-CoV	300/ND	ND	
17	Peptidomimetic – AG7088 derivative	SARS-CoV	200/ND	ND	
4a	Peptidomimetic	SARS-CoV	>25	ND	[145]
		MERS-CoV	>25	ND	
5a	Peptidomimetic	SARS-CoV	>25	ND	
		MERS-CoV	>25	ND	
6a	Peptidomimetic	SARS-CoV	>25	ND	
		MERS-CoV	>25	>100	
6b	Peptidomimetic	SARS-CoV	0.7±0.2	ND	
		MERS-CoV	2.4±0.3	1.4±0.0	
		229E	ND	4.3±0.1	
		OC43	ND	13.5±0.8	
		FIPV	ND	2.5±1.1	
6c	Peptidomimetic	SARS-CoV	0.5±0.1	ND	
		MERS-CoV	4.7±0.6	1.2±0.6	
		229E	ND	4.2±0.3	
		OC43	ND	16.8±0.3	
		FIPV	ND	1.9±0.2	
6d	Peptidomimetic	SARS-CoV	ND	ND	
		MERS-CoV	1.7±0.3	0.6±0.0	
		229E	ND	2.0±0.2	
		OC43	ND	17.7±1.6	
		FIPV	ND	1.1±0.3	

Continued

Table 1. Continued

Compound	Type of compound/ source	Virus	IC ₅₀ (μM)	EC ₅₀ (μM)	References
9a	Structure-based design	MERS SARS FIPV	0.6 2.1 0.8	ND	[103]
10a	Structure-based design	MERS SARS FIPV	0.4 5.1 2.4	0.5 0.6 1.5	
9b	Structure-based design	MERS SARS FIPV	0.7 28.8 3.5	ND	
10b	Structure-based design	MERS SARS FIPV	0.6 42.1 2.3	ND	
9c	Structure-based design	MERS SARS FIPV	0.8 5.2 1.6	ND	
10c	Structure-based design	MERS SARS FIPV	0.7 6.3 2.1	0.8 1.0 0.1	
9d	Structure-based design	MERS SARS FIPV	0.7 3.9 0.8	ND	
10d	Structure-based design	MERS SARS FIPV	0.9 4.3 1.1	ND	
9e	Structure-based design	MERS SARS FIPV	6.1 5.5 5.5	ND	
10e	Structure-based design	MERS SARS FIPV	7.5 4.1 6.7	ND	
9f	Structure-based design	MERS SARS FIPV	0.6 3.2 1.3	ND	
10f	Structure-based design	MERS SARS FIPV	0.5 8.8 1.1	ND	
11a	Structure-based design	SARS-CoV HCoV-NL63	1.95±0.24 >50	2.0±0.2	[106]
11n	Structure-based design	SARS-CoV HCoV-NL63	0.33±0.04 1.08±0.09	7.2±0.2	
11r	Structure-based design	SARS-CoV HCoV-NL63	0.71±0.36 12.27±3.56	1.4±0.1	
11s	Structure-based design	SARS-CoV HCoV-NL63	0.24±0.08 1.37±0.35	1.9±0.1	
11t	Structure-based design	SARS-CoV HCoV-NL63	1.44±0.4- 3.43±2.45	6.7±0.2	
11u	Structure-based design	SARS-CoV HCoV-NL63	1.27±0.34 5.41±2.31	3.6±0.1	

Continued

Table 1. Continued

Compound	Type of compound/ source	Virus	IC ₅₀ (μM)	EC ₅₀ (μM)	References
48	Peptidomimetic-decahydroisoquinolin derivatives	SARS-CoV	68	ND	[109]
41	Peptidomimetic-decahydroisoquinolin derivatives	SARS-CoV	63	ND	
49	Peptidomimetic-decahydroisoquinolin derivatives	SARS-CoV	49	ND	
6n	Peptidomimetic -serine derivatives	SARS-CoV	85	ND	[1]
6o	Peptidomimetic -serine derivatives	SARS-CoV	65	ND	
4 (SK80)	Phenylisoserine derivatives	SARS-CoV	43	ND	[111]
10	Peptidomimetic – phenylisoserine derivatives	SARS-CoV	85	ND	
17	Peptidomimetic – phenylisoserine derivatives	SARS-CoV	75	ND	
18	peptidomimetic - phenylisoserine derivatives	SARS-CoV	65	ND	
12	Peptidomimetic – phenylisoserine derivatives	SARS-CoV	65	ND	
Ac-Val-Leu-NHCH(CH ₂ CH ₂ CON(CH ₃) ₂)-CHO	Peptidomimetic	SARS-CoV	~6	ND	[108]
Ac-Ser-Ala-Val-Leu-NHCH(CH ₂ CH ₂ CON(CH ₃) ₂)-CHO	Peptidomimetic	SARS-CoV	37	ND	
Ac-Thr-Ser-Ala-Val-Leu-NHCH(CH ₂ CH ₂ CON(CH ₃) ₂)-CHO	Peptidomimetic	SARS-CoV	26	ND	
Cinanserin	<i>In silico</i> design	SARS-CoV HCoV-229E	4.92 4.68	ND	[112]
Cinanserin hydrochloride	<i>In silico</i> design	SARS-CoV HCoV-229E	5.05 5.68	ND	
53	Non-covalent	SARS-CoV	10	ND	[113]
54	Non-covalent	SARS-CoV	5.5	ND	
56	Non-covalent	SARS-CoV	45	ND	

Continued

Table 1. Continued

Compound	Type of compound/ source	Virus	IC ₅₀ (μM)	EC ₅₀ (μM)	References
10c	Non-covalent	SARS-CoV	11	ND	[114]
13a	Non-covalent	SARS-CoV	7.72	ND	
13b	Non-covalent	SARS-CoV	25.3	ND	
13c	Non-covalent	SARS-CoV	6.9	ND	
13d	Non-covalent	SARS-CoV	4.1	ND	
13e	Non-covalent	SARS-CoV	22.5	ND	
13f	Non-covalent	SARS-CoV	9.1	ND	
13g	Non-covalent	SARS-CoV	3.8	ND	
13k	Non-covalent	SARS-CoV	26	ND	
16a	Non-covalent	SARS-CoV	2.9	ND	
16b	Non-covalent	SARS-CoV	3.6	ND	
16c	Non-covalent	SARS-CoV	13.3	ND	
16d	Non-covalent	SARS-CoV	3.4	ND	
16e	Non-covalent	SARS-CoV	4.1	ND	
16f	Non-covalent	SARS-CoV	8.1	ND	
16g	Non-covalent	SARS-CoV	22.1	ND	
16i	Non-covalent	SARS-CoV	10.3	ND	
16j	Non-covalent	SARS-CoV	2.1	ND	
16k	Non-covalent	SARS-CoV	1.5	ND	
17a	Non-covalent	SARS-CoV	0.051	ND	
17b	Non-covalent	SARS-CoV	0.97	ND	
17c	Non-covalent	SARS-CoV	0.70	ND	
17d	Non-covalent	SARS-CoV	2.0	ND	
17e	Non-covalent	SARS-CoV	15.5	ND	

ND, not determined.

measured the IC₅₀ for nsp5 of MERS-CoV, SARS-CoV and feline infectious peritonitis virus (FIPV) [103]. Interestingly, some of the compounds had varying degrees of activity against the different proteases. For the compounds tested, the MERS-CoV IC₅₀ ranged from 0.5 to 7.5 μM, FIPV ranged from 0.8 to 6.7 μM, but SARS-CoV was more divergent with ranges from 2.1 to 42.1 μM. They then tested the EC₅₀ of two compounds with MERS-CoV, MHV and FIPV and showed a range of 0.5–1.5 μM across the three viruses. However, SARS-CoV was not tested for EC₅₀ and it had the highest, most divergent IC₅₀ values among the viruses tested. These data probably indicate differences in binding within the active site of each of these proteases and highlights the need to test inhibitors against multiple CoV proteases. This finding is consistent with both variations in the active sites between the CoV proteases (Fig. 2) as well as

known differences in cleavage site sequences in pp1ab [67]. Consistent with numerous other studies evaluating inhibitors bound to crystallized nsp5 proteases, coordination between the active site inhibitor and subtle variations in the residues lining the pocket may be impacting the strength of binding and fit of the inhibitor [75, 104, 105]. In another study using a structure-based design, Zhang *et al.* were able to develop several α-ketoamides that have a glutamine lactam at the P1 site that mimics the glutamine in the P1 position of the nsp5 consensus cleavage site [106]. This strategy resulted in the design of six compound derivatives with EC₅₀ values of <10 μM against SARS-CoV replicons and cross-reactivity with the enterovirus EV-A71 and CVB3 3C proteases. This cross-reactivity would allow for development of more broad-spectrum antivirals that can be tested using structure-activity

relationships (SARs) to make the compound more specific and potent to certain viral proteases.

There have been challenges to screening compounds *in vitro* with tagged nsp5. While purifying N-terminally tagged nsp5 for testing peptidomimetic inhibitors, Akaji *et al.* identified an internal cleavage site in the SARS-CoV nsp5 at R188-Q189 that resulted in loss of enzyme activity that could have obscured the results of inhibition activity. They introduced an R188I mutation that prevents internal cleavage during *in vitro* screening and then used a structure-based design of inhibitors. Through several follow-up studies, they have identified inhibitors based on several scaffolds including aldehyde, decahydroisoquinolin, and serine or phenylisoserine derivatives. Using SAR analysis, they were able to identify compounds with IC_{50} of $<100\ \mu\text{M}$ [107–111]. In future studies, this R188I substitution will need to be used to screen compounds if tagging the N terminus of nsp5.

There has been some computational analysis of potential nsp5 inhibitors. Chen *et al.* screened the MDL-CMC database for inhibitors that bound the nsp5 active site *in silico* [112]. They then identified 10 compounds and were able to show that cinanserine had antiviral activity against nsp5 from both SARS-CoV and HCoV-229E, as well as the HCoV-229E replicon cells, and during infections with SARS-CoV and HCoV-229E. However, a subsequent study showed no inhibition of the SARS-CoV, HCoV-229E or MHV nsp5 proteases up to $100\ \mu\text{M}$ of cinanserine [113]. Jacobs *et al.* used a high-throughput assay to test several non-covalent nsp5 inhibitors and identified several with an IC_{50} of $>100\ \mu\text{M}$ and showed that ML-188 [also called 16-(R)] had an EC_{50} between 12.9 ± 0.7 and $13.4\pm 1.0\ \mu\text{M}$. Subsequently, this group then screened additional compounds and performed SAR studies to improve the efficacy of the compounds toward the SARS-CoV nsp5 protease [114].

Novel potential targets for future nsp5 inhibitor design

There has been limited work on testing for resistance to these active-site inhibitors. However, one study showed that three substitutions arose that block inhibition of CE-5 [94]. Of the three substitutions, two were in domain 1 and the other was in domain 3 (Fig. 4). Interestingly, the substitutions in domain 1 were not in the active site but were located above the active site. This, combined with studies demonstrating that temperature-sensitive mutants were capable of gaining function through substitutions in distant portion of the proteins [85, 115], suggests that (1) we should expect that resistance mutations will arise to these active site inhibitors, and (2) those changes that confer resistance may be in one of many different locations throughout the protease due to the complex interactions within the protease that are required for enzymatic function and between the protease and host and viral proteins regulating its function allosterically. Therefore, targeting multiple regions of the protease may be a better strategy than using a single therapeutic that targets the active site. Therefore, there remain many additional potential

approaches which may be used to inhibit nsp5 as highlighted in Fig. 5, which include protein folding and stability, dimerization, and allosteric interactions with host and viral proteins.

Protein folding and stability

In order to be catalytically active, nsp5 has to be properly folded into the correct conformation after it is cleaved from the polyprotein. While it may not be possible to target specific folding of nsp5, there has been studies showing the use of protein folding inhibition in cancer treatment [116]. Since this would alter global protein folding, there may be challenges in delivery of such a compound. As groups are screening compounds *in silico*, it would be interesting to also look for allosteric inhibitors that bind to nsp5 and alter the conformation of the active site to preclude substrate binding or alter protein stability. The interdomain loop (IDL), which connects domains 2 and 3, is a structurally conserved region of the protease that is probably involved in substrate recognition [78, 117–119]. This region would be an interesting target for inhibition since altering the IDL could change the stability of the chymotrypsin-like fold or alter substrate recognition to decrease enzymatic activity. Other regions are known to be involved in protein stability and function. Through the study of nsp5 temperature-sensitive mutants and their second-site suppressor mutations in both MHV and OC43 [63, 85], it has been suggested that there are long-distance communication networks within the nsp5 structure. These nodes of the long-distance communication can be targeted by small molecules to alter the conformation and stability of nsp5.

Dimerization

Targeting dimerization of a protease is a strategy that has been explored with human immunodeficiency virus (HIV) [120–122]. The mechanism of inhibition is to design peptides that match the dimerization region that will then bind to the region and prevent the binding of a second protease molecule. Dimerization of nsp5 is critical for nsp5 function [123]. Based on the strategy used for the HIV protease, an N-terminal octapeptide of nsp5 has been designed and shown to inhibit dimerization [124, 125]. This peptide is likely to block interaction of the N-finger regions to prevent dimerization. Additional studies could be performed to enhance the potency of the peptide inhibitors of dimerization.

Allosteric interactions with host and viral proteins

Nsp5 interacts with other viral and host proteins to mediate virus replication and innate immune evasion and those interactions may also impact nsp5 function. Therefore, blocking these interactions could dampen viral replication. A recent study looked at interaction of 26 of 29 SARS-CoV-2 viral proteins with host proteins and showed that the catalytically dead nsp5 (C145A) interacts with proteins involved in the response to oxidative stress and mitochondrial matrix proteins [126]. Since nsp5 may cleave proteins that it interacts with and/or have short-lived interactions, these interactions may be difficult to assess by mass spectrometry. The proteins identified in the study are probably an underestimate of the

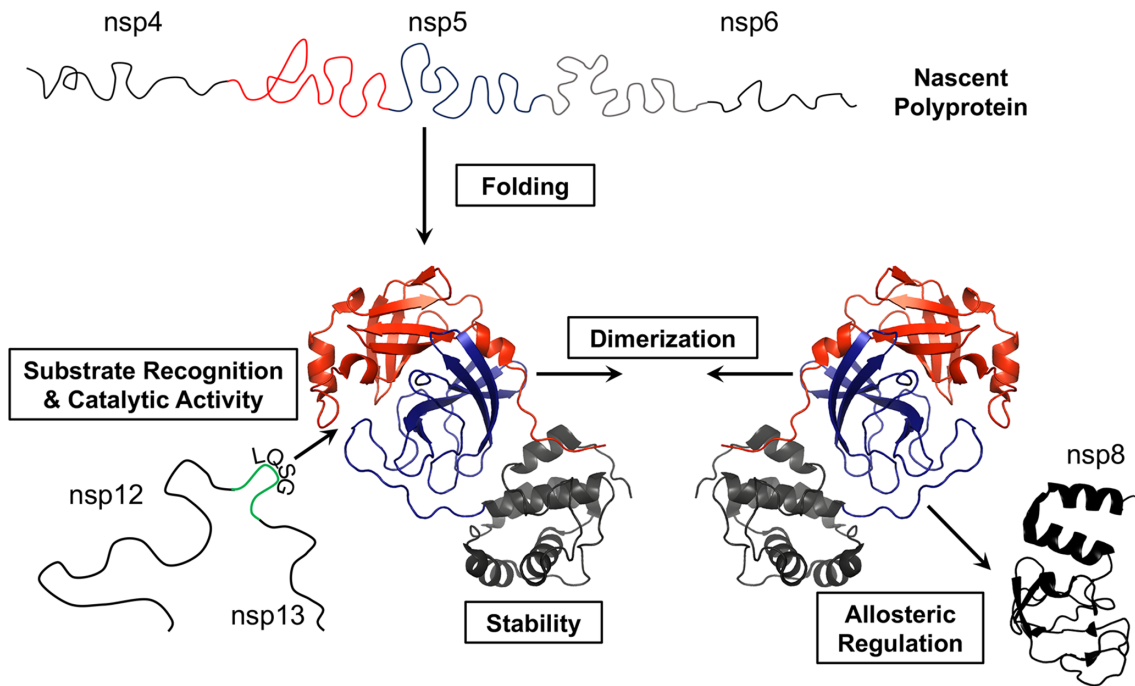


Fig. 5. Potential targets for coronavirus nsp5 therapeutic design. An outline of the key stages of nsp5 activity is shown. Crystal structures of monomeric SARS-CoV-2 nsp5 (PDB 6M2N) and the C-terminal region of the SARS-CoV-2 nsp8 structure (PDB 7BV1) are also depicted [146].

host proteins that nsp5 is interacting with. Additional yeast two-hybrid studies have shown that nsp5 interacts with other nsps, including nsp7 and nsp8 in SARS-CoV [127] or nsp7, nsp9 and nsp12 in PRRSV [128]. Since these studies were done with mature proteins, these interactions should be independent of cleavage and represent interactions that can be blocked using small molecules.

Several groups have shown that CoV nsp5 proteases cleave host targets in innate immune pathways. For example, porcine deltacoronavirus (PDCoV), porcine epidemic diarrhoea virus (PEDV) and FIPV nsp5 proteases cleave the nuclear factor- κ B (NF- κ B) essential modulator (NEMO) to antagonize type I IFNs [129–131]. PDCoV also cleaves STAT2 to inhibit activation of IFN-stimulated genes [132]. Recent studies with SARS-CoV-2 showed that nsp5 cleaved IRF3 and two innate immune proteins, NLRP12 and TAB1 [133]. It is likely that nsp5 may target other host proteins for cleavage, which may provide a unique and novel avenue for development of nsp5 inhibitors. Fragment-based design is a technique that is now being used to identify allosteric inhibitors [134–137]. For the HIV protease (PR), this approach was used and identified two allosteric sites that bound to PR and prevented access to the active site [136]. It was not determined whether these locations could block binding to other proteins. However, the fragment-based design technique has been used to disrupt allosteric interactions between the protease and helicase domains of hepatitis C virus NS3 [138]. It would be interesting to see what interactions could be disrupted using this method on CoV nsp5 proteases.

CONCLUSIONS

With the emergence of three coronaviruses in the last 20 years that cause significant disease and mortality, there remains a critical need for therapeutics for current and future emerging coronaviruses. As the SARS-CoV-2 pandemic approaches 25 million cases worldwide, the ability to have effective tools to limit COVID-19 disease and arrest the spread of this pandemic are paramount to a return to normality. Coronavirus protease nsp5 remains a key target for therapeutic design efforts and renewed interest should be given to find novel conserved structural and functional features of the protease that may be exploited. We have highlighted in this review an array of features that have and have not been extensively explored for therapeutic targets. It is our hope that an effective therapeutic with broad-spectrum activity against the nsp5 protease of a majority of coronaviruses can be developed to respond.

Funding Information

We recognize the Department of Biology at DeSales University (D.C.B.) and the Butler University Department of Biological Sciences and Holcomb Awards Committee (C.C.S.), and the Indiana Academy of Sciences (C.C.S.) for their funding and logistical support for our research.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

1. Corman VM, Muth D, Niemeyer D, Drosten C. Hosts and sources of endemic human coronaviruses. *Adv Virus Res* 2018;100:163–188.

2. Walsh EE, Shin JH, Falsey AR. Clinical impact of human coronaviruses 229E and OC43 infection in diverse adult populations. *J Infect Dis* 2013;208:1634–1642.
3. van der Hoek L, Sure K, Ihorst G, Stang A, Pyrc K et al. Croup is associated with the novel coronavirus NL63. *PLoS Med* 2005;2:e240.
4. Nickbakhsh S, Ho A, Marques DFP, McMenamin J, Gunson RN et al. Epidemiology of seasonal coronaviruses: establishing the context for the emergence of coronavirus disease 2019. *J Infect Dis* 2020;222:17–25.
5. Perlman S, Netland J. Coronaviruses post-SARS: update on replication and pathogenesis. *Nat Rev Microbiol* 2009;7:439–450.
6. Zhong NS, Zheng BJ, Li YM, Xie ZH et al. Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, people's Republic of China, in February, 2003. *Lancet* 2003;362:1353–1358.
7. R-H X, J-F H, Evans MR, Peng G-W, Field HE et al. Epidemiologic clues to SARS origin in China. *Emerg Infect Dis* 2004;10:1030–1037.
8. Lee N, Hui D, Wu A, Chan P, Cameron P et al. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 2003;348:1986–1994.
9. Cao W-C, de Vlas SJ, Richardus JH. The severe acute respiratory syndrome epidemic in mainland China dissected. *Infect Dis Rep* 2011;3:2.
10. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 2012;367:1814–1820.
11. Choi WS, Kang C-I, Kim Y, Choi J-P, Joh JS et al. Clinical presentation and outcomes of middle East respiratory syndrome in the Republic of Korea. *Infect Chemother* 2016;48:118–126.
12. Zumla A, Hui DS, Perlman S. Middle East respiratory syndrome. *The Lancet* 2015;386:995–1007.
13. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. *Nat Rev Microbiol* 2016;14:523–534.
14. Drosten C, Meyer B, Müller MA, Corman VM, Al-Masri M et al. Transmission of MERS-Coronavirus in household contacts. *N Engl J Med* 2014;371:828–835.
15. Coleman CM, Frieman MB. Coronaviruses: important emerging human pathogens. *J Virol* 2014;88:5209–5212.
16. Zhu N, Zhang D, Wang W, Li X, Yang B et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med* 2020;382:727–733.
17. Li Q, Guan X, Wu P, Wang X, Zhou L et al. Early transmission dynamics in Wuhan, China, of novel Coronavirus-Infected pneumonia. *N Engl J Med* 2020;382:1199–1207.
18. Sohrabi C, Alsafi Z, O'Neill N, Khan M, Kerwan A et al. World Health organization declares global emergency: a review of the 2019 novel coronavirus (COVID-19). *Int J Surg* 2020;76:71–76.
19. Chu H, Chan JF-W, Yuen TT-T, Shuai H, Yuan S et al. Comparative tropism, replication kinetics, and cell damage profiling of SARS-CoV-2 and SARS-CoV with implications for clinical manifestations, transmissibility, and laboratory studies of COVID-19: an observational study. *The Lancet Microbe* 2020;1:e14–e23.
20. Park SE. Epidemiology, virology, and clinical features of severe acute respiratory syndrome -coronavirus-2 (SARS-CoV-2; coronavirus Disease-19). *Clin Exp Pediatr* 2020;63:119–124.
21. Chen J. Pathogenicity and transmissibility of 2019-nCoV—A quick overview and comparison with other emerging viruses. *Microbes Infect* 2020;22:69–71.
22. Wang Y, Wang Y, Chen Y, Qin Q. Unique epidemiological and clinical features of the emerging 2019 novel coronavirus pneumonia (COVID-19) implicate special control measures. *J Med Virol* 2020.
23. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J Autoimmun* 2020;109:102433.
24. Wang D, Hu B, Hu C, Zhu F, Liu X et al. Clinical characteristics of 138 hospitalized patients with 2019 novel Coronavirus-Infected pneumonia in Wuhan, China. *JAMA* 2020;323:1061–1069.
25. Biswas A, Bhattacharjee U, Chakrabarti AK, Tewari DN, Banu H et al. Emergence of novel coronavirus and COVID-19: whether to stay or die out? *Crit Rev Microbiol* 2020:1–12.
26. De Sabato L, Lelli D, Faccin F, Canziani S, Di Bartolo I et al. Full genome characterization of two novel Alpha-coronavirus species from Italian bats. *Virus Res* 2019;260:60–66.
27. Zhang Y-Z, Holmes EC. A genomic perspective on the origin and emergence of SARS-CoV-2. *Cell* 2020;181:223–227.
28. Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020;579:270–273.
29. Yousefi B, Valizadeh S, Ghaffari H, Vahedi A, Karbalaeei M et al. A global treatments for coronaviruses including COVID-19. *J Cell Physiol* 2020.
30. Li G, De Clercq E. Therapeutic options for the 2019 novel coronavirus (2019-nCoV). *Nat Rev Drug Discov* 2020;19:149–150.
31. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol* 2015;1282:1–23.
32. Bosch BJ, van der Zee R, de Haan CAM, Rottier PJM. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J Virol* 2003;77:8801–8811.
33. Collins AR, Knobler RL, Powell H, Buchmeier MJ. Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. *Virology* 1982;119:358–371.
34. Yeager CL, Ashmun RA, Williams RK, Cardellicchio CB, Shapiro LH et al. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* 1992;357:420–422.
35. Wu K, Li W, Peng G, Li F. Crystal structure of NL63 respiratory coronavirus receptor-binding domain complexed with its human receptor. *Proc Natl Acad Sci U S A* 2009;106:19970–19974.
36. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003;426:450–454.
37. Raj VS, Mou H, Smits SL, Dekkers DHW, Müller MA et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* 2013;495:251–254.
38. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J Virol* 2020;94.
39. Glowacka I, Bertram S, Müller MA, Allen P, Soilleux E et al. Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. *J Virol* 2011;85:4122–4134.
40. Brierley I, Digard P, Inglis SC. Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot. *Cell* 1989;57:537–547.
41. Lee H-J, Shieh C-K, Gorbalenya AE, Koonin EV, La Monica N et al. The complete sequence (22 kilobases) of murine coronavirus gene 1 encoding the putative proteases and RNA polymerase. *Virology* 1991;180:567–582.
42. Ziebuhr J, Gorbalenya AE, Snijder EJ. Virus-encoded proteinases and proteolytic processing in the Nidovirales. *J Gen Virol* 2000;81:853–879.
43. Gorbalenya AE, Koonin EV, Donchenko AP, Blinov VM. Coronavirus genome: prediction of putative functional domains in the non-structural polyprotein by comparative amino acid sequence analysis. *Nucleic Acids Res* 1989;17:4847–4861.
44. de Wilde AH, Snijder EJ, Kikkert M, van Hemert MJ. Host factors in coronavirus replication. *Curr Top Microbiol Immunol* 2018;419:1–42.

45. Knoops K, Kikkert M, Worm SHEvanden, Zevenhoven-Dobbe JC, van der Meer Y et al. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biol* 2008;6:e226.
46. Angelini MM, Akhlaghpour M, Neuman BW, Buchmeier MJ. Severe acute respiratory syndrome coronavirus nonstructural proteins 3, 4, and 6 induce double-membrane vesicles. *MBio* 2013;4.
47. Gadlage MJ, Sparks JS, Beachboard DC, Cox RG, Doyle JD et al. Murine hepatitis virus nonstructural protein 4 regulates virus-induced membrane modifications and replication complex function. *J Virol* 2010;84:280–290.
48. Beachboard DC, Anderson-Daniels JM, Denison MR. Mutations across murine hepatitis virus NSP4 alter virus fitness and membrane modifications. *J Virol* 2015;89:2080–2089.
49. Sawicki SG, Sawicki DL, Siddell SG. A contemporary view of coronavirus transcription. *J Virol* 2007;81:20–29.
50. Vennema H, Heijnen L, Zijderveld A, Horzinek MC, Spaan WJ. Intracellular transport of recombinant coronavirus spike proteins: implications for virus assembly. *J Virol* 1990;64:339–346.
51. Krijnse-Locker J, Ericsson M, Rottier PJ, Griffiths G. Characterization of the budding compartment of mouse hepatitis virus: evidence that transport from the rER to the Golgi complex requires only one vesicular transport step. *J Cell Biol* 1994;124:55–70.
52. Tooze J, Tooze S, Warren G. Replication of coronavirus MHV-A59 in sac- cells: determination of the first site of budding of progeny virions. *Eur J Cell Biol* 1984;33:281–293.
53. de Haan CAM, Rottier PJM. Molecular interactions in the assembly of coronaviruses. *Adv Virus Res* 2005;64:165–230.
54. Siu YL, Teoh KT, Lo J, Chan CM, Kien F et al. The M, E, and N structural proteins of the severe acute respiratory syndrome coronavirus are required for efficient assembly, trafficking, and release of virus-like particles. *J Virol* 2008;82:11318–11330.
55. Gallagher TM, Buchmeier MJ, Perlman S. Cell receptor-independent infection by a neurotropic murine coronavirus. *Virology* 1992;191:517–522.
56. Sparks JS, Donaldson EF, Lu X, Baric RS, Denison MR. A novel mutation in murine hepatitis virus NSP5, the viral 3C-like proteinase, causes temperature-sensitive defects in viral growth and protein processing. *J Virol* 2008;82:5999–6008.
57. Lu Y, Lu X, Denison MR. Identification and characterization of a serine-like proteinase of the murine coronavirus MHV-A59. *J Virol* 1995;69:3554–3559.
58. Lu Y, Denison MR. Determinants of mouse hepatitis virus 3C-like proteinase activity. *Virology* 1997;230:335–342.
59. Baker SC, Yokomori K, Dong S, Carlisle R, Gorbalenya AE et al. Identification of the catalytic sites of a papain-like cysteine proteinase of murine coronavirus. *J Virol* 1993;67:6056–6063.
60. Chen Z, Wang Y, Ratia K, Mesecar AD, Wilkinson KD et al. Proteolytic processing and deubiquitinating activity of papain-like proteases of human coronavirus NL63. *J Virol* 2007;81:6007–6018.
61. Ratia K, Saikatendu KS, Santarsiero BD, Barretto N, Baker SC et al. Severe acute respiratory syndrome coronavirus papain-like protease: structure of a viral deubiquitinating enzyme. *Proc Natl Acad Sci U S A* 2006;103:5717–5722.
62. Mielech AM, Chen Y, Mesecar AD, Baker SC. Nidovirus papain-like proteases: multifunctional enzymes with protease, deubiquitinating and delSgylating activities. *Virus Res* 2014;194:184–190.
63. Stobart CC, Sexton NR, Munjal H, Lu X, Molland KL et al. Chimeric exchange of coronavirus NSP5 proteases (3CLpro) identifies common and divergent regulatory determinants of protease activity. *J Virol* 2013;87:12611–12618.
64. Lu X, Lu Y, Denison MR. Intracellular and in vitro-translated 27-kDa proteins contain the 3C-like proteinase activity of the coronavirus MHV-A59. *Virology* 1996;222:375–382.
65. Anand K, Ziebuhr J, Wadhvani P, Mesters JR, Hilgenfeld R. Coronavirus main proteinase (3CLpro) structure: basis for design of anti-SARS drugs. *Science* 2003;300:1763–1767.
66. Xue X, Yu H, Yang H, Xue F, Wu Z et al. Structures of two coronavirus main proteases: implications for substrate binding and antiviral drug design. *J Virol* 2008;82:2515–2527.
67. Grum-Tokars V, Ratia K, Begaye A, Baker SC, Mesecar AD. Evaluating the 3C-like protease activity of SARS-coronavirus: recommendations for standardized assays for drug discovery. *Virus Res* 2008;133:63–73.
68. Hsu M-F, Kuo C-J, Chang K-T, Chang H-C, Chou C-C et al. Mechanism of the maturation process of SARS-CoV 3CL protease. *J Biol Chem* 2005;280:31257–31266.
69. Hegyi A, Ziebuhr J. Conservation of substrate specificities among coronavirus main proteases. *J Gen Virol* 2002;83:595–599.
70. Hegyi A, Friebe A, Gorbalenya AE, Ziebuhr J. Mutational analysis of the active centre of coronavirus 3C-like proteases. *J Gen Virol* 2002;83:581–593.
71. Shi J, Song J. The catalysis of the SARS 3C-like protease is under extensive regulation by its extra domain. *FEBS J* 2006;273:1035–1045.
72. Shi J, Sivaraman J, Song J. Mechanism for controlling the dimer-monomer switch and coupling dimerization to catalysis of the severe acute respiratory syndrome coronavirus 3C-like protease. *J Virol* 2008;82:4620–4629.
73. Anand K, Palm GJ, Mesters JR, Siddell SG, Ziebuhr J. Structure of coronavirus main proteinase reveals combination of a chymotrypsin fold with an extra alpha-helical domain. *Embo J* 2002;21:3213–3224.
74. Bacha U, Barrila J, Gabelli SB, Kiso Y, Mario Amzel L et al. Development of broad-spectrum halomethyl ketone inhibitors against coronavirus main protease 3CL^{pro}. *Chem Biol Drug Des* 2008;72:34–49.
75. Tomar S, Johnston ML, John SES, Osswald HL, Nyalapatla PR et al. Ligand-Induced dimerization of middle East respiratory syndrome (MERS) coronavirus NSP5 protease (3CLpro) implications for NSP5 regulation and the development of antivirals. *J Biol Chem* 2015;290:19403–19422.
76. Cheng S-C, Chang G-G, Chou C-Y. Mutation of Glu-166 blocks the substrate-induced dimerization of SARS coronavirus main protease. *Biophys J* 2010;98:1327–1336.
77. Chen S, Chen L, Tan J, Chen J, Du L et al. Severe acute respiratory syndrome coronavirus 3C-like proteinase N terminus is indispensable for proteolytic activity but not for enzyme dimerization. Biochemical and thermodynamic investigation in conjunction with molecular dynamics simulations. *J Biol Chem* 2005;280:164–173.
78. Zhang L, Lin D, Sun X, Curth U, Drosten C et al. Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α -ketoamide inhibitors. *Science* 2020;368:409–412.
79. Kanjanahaluthai A, Baker SC. Identification of mouse hepatitis virus papain-like proteinase 2 activity. *J Virol* 2000;74:7911–7921.
80. Donaldson EF, Graham RL, Sims AC, Denison MR, Baric RS. Analysis of murine hepatitis virus strain A59 temperature-sensitive mutant TS-LA6 suggests that nsp10 plays a critical role in poly-protein processing. *J Virol* 2007;81:7086–7098.
81. Li C, Qi Y, Teng X, Yang Z, Wei P et al. Maturation mechanism of severe acute respiratory syndrome (SARS) coronavirus 3C-like proteinase. *J Biol Chem* 2010;285:28134–28140.
82. Chen S, Jonas F, Shen C, Higenfeld R, Higenfeld R. Liberation of SARS-CoV main protease from the viral polyprotein: N-terminal autocleavage does not depend on the mature dimerization mode. *Protein Cell* 2010;1:59–74.
83. Krichel B, Falke S, Hilgenfeld R, Redecke L, Uetrecht C. Processing of the SARS-CoV pp1a/ab nsp7–10 region. *Biochem J* 2020;477:1009–1019.

84. Deming DJ, Graham RL, Denison MR, Baric RS. Processing of open reading frame 1A replicase proteins nsp7 to nsp10 in murine hepatitis virus strain A59 replication. *J Virol* 2007;81:10280–10291.
85. Stobart CC, Lee AS, Lu X, Denison MR. Temperature-sensitive mutants and revertants in the coronavirus nonstructural protein 5 protease (3CLpro) define residues involved in long-distance communication and regulation of protease activity. *J Virol* 2012;86:4801–4810.
86. Stokes HL, Baliji S, Hui CG, Sawicki SG, Baker SC et al. A new cistron in the murine hepatitis virus replicase gene. *J Virol* 2010;84:10148–10158.
87. Ahn D-G, Shin H-J, Kim M-H, Lee S, Kim H-S et al. Current status of epidemiology, diagnosis, therapeutics, and vaccines for novel coronavirus disease 2019 (COVID-19). *J Microbiol Biotechnol* 2020;30:313–324.
88. Koirala A, Joo YJ, Khatami A, Chiu C, Britton PN. Vaccines for COVID-19: the current state of play. *Paediatr Respir Rev* 2020.
89. Pillaiyar T, Manickam M, Namasivayam V, Hayashi Y, Jung S-H. An overview of severe acute respiratory Syndrome-Coronavirus (SARS-CoV) 3CL protease inhibitors: peptidomimetics and small molecule chemotherapy. *J Med Chem* 2016;59:6595–6628.
90. Pillaiyar T, Meenakshisundaram S, Manickam M. Recent discovery and development of inhibitors targeting coronaviruses. *Drug Discov Today* 2020;25:668–688.
91. Khan SA, Zia K, Ashraf S, Uddin R, Ul-Haq Z. Identification of chymotrypsin-like protease inhibitors of SARS-CoV-2 via integrated computational approach. *J Biomol Struct Dyn* 2020;0:1–10.
92. Kanhed AM, Patel DV, Teli DM, Patel NR, Chhabria MT et al. Identification of potential M^{pro} inhibitors for the treatment of COVID-19 by using systematic virtual screening approach. *Mol Divers* 2020;10.
93. C-Y W, King K-Y, Kuo C-J, Fang J-M, Y-T W et al. Stable benzotriazole esters as mechanism-based inactivators of the severe acute respiratory syndrome 3CL protease. *Chemistry & Biology* 2006;13:261–268.
94. Deng X, StJohn SE, Osswald HL, O'Brien A, Banach BS et al. Coronaviruses resistant to a 3C-like protease inhibitor are attenuated for replication and pathogenesis, revealing a low genetic barrier but high fitness cost of resistance. *J Virol* 2014;88:11886–11898.
95. Ghosh AK, Gong G, Grum-Tokars V, Mulhearn DC, Baker SC et al. Design, synthesis and antiviral efficacy of a series of potent chloropyridyl ester-derived SARS-CoV 3CLpro inhibitors. *Bioorg Med Chem Lett* 2008;18:5684–5688.
96. Kilianski A, Mielech AM, Deng X, Baker SC. Assessing activity and inhibition of middle East respiratory syndrome coronavirus papain-like and 3C-like proteases using luciferase-based biosensors. *J Virol* 2013;87:11955–11962.
97. Agnihothram S, Yount BL, Donaldson EF, Huynh J, Menachery VD et al. A mouse model for Betacoronavirus subgroup 2C using a bat coronavirus strain HKU5 variant. *mBio* 2014;5.
98. Ghosh AK, Xi K, Grum-Tokars V, Xu X, Ratia K et al. Structure-based design, synthesis, and biological evaluation of peptidomimetic SARS-CoV 3CLpro inhibitors. *Bioorg Med Chem Lett* 2007;17:5876–5880.
99. Ghosh AK, Xi K, Ratia K, Santarsiero BD, Fu W et al. Design and synthesis of peptidomimetic severe acute respiratory syndrome chymotrypsin-like protease inhibitors. *J Med Chem* 2005;48:6767–6771.
100. Matthews DA, Patick AK, Baker RO, Brothers MA, Dragovich PS et al. *In vitro* antiviral activity of human rhinovirus 3c protease inhibitors against the SARS coronavirus [Internet]. learning from SARS: preparing for the next disease outbreak: workshop summary. *National Academies Press* 2004.
101. Shie J-J, Fang J-M, Kuo T-H, Kuo C-J, Liang P-H et al. Inhibition of the severe acute respiratory syndrome 3CL protease by peptidomimetic α,β -unsaturated esters. *Bioorg Med Chem* 2005;13:5240–5252.
102. Kumar V, Shin JS, Shie J-J, Ku KB, Kim C et al. Identification and evaluation of potent middle East respiratory syndrome coronavirus (MERS-CoV) 3CL pro inhibitors. *Antiviral Res* 2017;141:101–106.
103. Galasiti Kankanamalage AC, Kim Y, Damalanka VC, Rathnayake AD, Fehr AR et al. Structure-guided design of potent and permeable inhibitors of MERS coronavirus 3CL protease that utilize a piperidine moiety as a novel design element. *Eur J Med Chem* 2018;150:334–346.
104. Cui W, Cui S, Chen C, Chen X, Wang Z et al. The crystal structure of main protease from mouse hepatitis virus A59 in complex with an inhibitor. *Biochem Biophys Res Commun* 2019;511:794–799.
105. Akaji K, Konno H, Mitsui H, Teruya K, Shimamoto Y et al. Structure-Based design, synthesis, and evaluation of peptide-mimetic SARS 3CL protease inhibitors. *J Med Chem* 2011;54:7962–7973.
106. Zhang L, Lin D, Kusov Y, Nian Y, Ma Q et al. α -Ketoamides as broad-spectrum inhibitors of coronavirus and enterovirus replication: structure-based design, synthesis, and activity assessment. *J Med Chem* 2020;63:4562–4578.
107. Akaji K, Konno H, Mitsui H, Teruya K, Shimamoto Y et al. Structure-based design, synthesis, and evaluation of peptide-mimetic SARS 3CL protease inhibitors. *J Med Chem* 2011;54:7962–7973.
108. Akaji K, Konno H, Onozuka M, Makino A, Saito H et al. Evaluation of peptide-aldehyde inhibitors using R188I mutant of SARS 3CL protease as a proteolysis-resistant mutant. *Bioorg Med Chem* 2008;16:9400–9408.
109. Shimamoto Y, Hattori Y, Kobayashi K, Teruya K, Sanjoh A et al. Fused-ring structure of decahydroisoquinolin as a novel scaffold for SARS 3CL protease inhibitors. *Bioorg Med Chem* 2015;23:876–890.
110. Konno H, Wakabayashi M, Takanuma D, Saito Y, Akaji K. Design and synthesis of a series of serine derivatives as small molecule inhibitors of the SARS coronavirus 3CL protease. *Bioorg Med Chem* 2016;24:1241–1254.
111. Konno H, Onuma T, Nitani I, Wakabayashi M, Yano S et al. Synthesis and evaluation of phenylisoserine derivatives for the SARS-CoV 3CL protease inhibitor. *Bioorg Med Chem Lett* 2017;27:2746–2751.
112. Chen L, Gui C, Luo X, Yang Q, Günther S et al. Cinanserin is an inhibitor of the 3C-like proteinase of severe acute respiratory syndrome coronavirus and strongly reduces virus replication in vitro. *J Virol* 2005;79:7095–7103.
113. Jacobs J, Grum-Tokars V, Zhou Y, Turlington M, Saldanha SA et al. Discovery, synthesis, and structure-based optimization of a series of N-(tert-butyl)-2-(N-arylamido)-2-(pyridin-3-yl) acetamides (ML188) as potent noncovalent small molecule inhibitors of the severe acute respiratory syndrome coronavirus (SARS-CoV) 3CL protease. *J Med Chem* 2013;56:534–546.
114. Turlington M, Chun A, Tomar S, Eggler A, Grum-Tokars V et al. Discovery of N-(benzo[1,2,3]triazol-1-yl)-N-(benzyl)acetamido phenyl carboxamides as severe acute respiratory syndrome coronavirus (SARS-CoV) 3CLpro inhibitors: identification of ML300 and noncovalent nanomolar inhibitors with an induced-fit binding. *Bioorg Med Chem Lett* 2013;23:6172–6177.
115. Sparks JS, Donaldson EF, Lu X, Baric RS, Denison MR. A novel mutation in murine hepatitis virus NSP5, the viral 3C-like proteinase, causes temperature-sensitive defects in viral growth and protein processing. *J Virol* 2008;82:5999–6008.
116. Boschelli F, Golas JM, Petersen R, Lau V, Chen L et al. A cell-based screen for inhibitors of protein folding and degradation. *Cell Stress Chaperones* 2010;15:913–927.
117. Muramatsu T, Takemoto C, Kim Y-T, Wang H, Nishii W et al. SARS-Cov 3CL protease cleaves its C-terminal autoprocessing site by novel subsite cooperativity. *Proc Natl Acad Sci U S A* 2016;113:12997–13002.
118. Gimeno A, Mestres-Truyol J, Ojeda-Montes MJ, Macip G, Saldivar-Espinoza B et al. Prediction of novel inhibitors of the main protease (M-pro) of SARS-CoV-2 through consensus docking and drug reposition. *Int J Mol Sci* 2020;21:3793.

119. Jo S, Kim S, Shin DH, Kim M-S. Inhibition of SARS-CoV 3CL protease by flavonoids. *J Enzyme Inhib Med Chem* 2020;35:145–151.
120. Boggetto N, Reboud-Ravaux M. Dimerization inhibitors of HIV-1 protease. *Biol Chem* 2002;383:1321–1324.
121. Schramm HJ, Nakashima H, Schramm W, Wakayama H, Yamamoto N. HIV-1 reproduction is inhibited by peptides derived from the N- and C-termini of HIV-1 protease. *Biochem Biophys Res Commun* 1991;179:847–851.
122. Zutshi R, Franciskovich J, Shultz M, Schweitzer B, Bishop P et al. Targeting the Dimerization Interface of HIV-1 Protease: Inhibition with Cross-Linked Interfacial Peptides. *J Am Chem Soc* 1997;119:4841–4845.
123. Goyal B, Goyal D. Targeting the dimerization of the main protease of coronaviruses: a potential broad-spectrum therapeutic strategy. *ACS Comb Sci* 2020;22:297–305.
124. Ding L, Zhang X-X, Wei P, Fan K, Lai L. The interaction between severe acute respiratory syndrome coronavirus 3C-like proteinase and a dimeric inhibitor by capillary electrophoresis. *Anal Biochem* 2005;343:159–165.
125. Wei P, Fan K, Chen H, Ma L, Huang C et al. The N-terminal octapeptide acts as a dimerization inhibitor of SARS coronavirus 3C-like proteinase. *Biochem Biophys Res Commun* 2006;339:865–872.
126. Gordon DE, Jang GM, Bouhaddou M, Xu J, Obernier K et al. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 2020;583:459–468.
127. von BA, Teepe C, Simpson JC, Pepperkok R, Friedel CC et al. Analysis of Intraviral protein-protein interactions of the SARS coronavirus ORFome. *PLoS One*. 2007;2:e459.
128. Song J, Liu Y, Gao P, Hu Y, Chai Y et al. Mapping the nonstructural protein interaction network of porcine reproductive and respiratory syndrome virus. *J Virol* 2018;92.
129. Zhu X, Fang L, Wang D, Yang Y, Chen J et al. Porcine deltacoronavirus NSP5 inhibits interferon- β production through the cleavage of NEMO. *Virology* 2017;502:33–38.
130. Chen S, Tian J, Li Z, Kang H, Zhang J et al. Feline infectious peritonitis virus NSP5 inhibits type I interferon production by cleaving NEMO at multiple sites. *Viruses* 2019;12:43.
131. Wang D, Fang L, Shi Y, Zhang H, Gao L et al. Porcine epidemic diarrhea virus 3C-like protease regulates its interferon antagonism by cleaving NEMO. *J Virol* 2016;90:2090–2101.
132. Zhu X, Wang D, Zhou J, Pan T, Chen J et al. Porcine deltacoronavirus NSP5 antagonizes type I interferon signaling by cleaving STAT2. *J Virol* 2017;91.
133. Moustaqil M, Ollivier E, Chiu H-P, Tol SV, Rudolffi-Soto P et al. SARS-CoV-2 proteases cleave IRF3 and critical modulators of inflammatory pathways (NLRP12 and TAB1): implications for disease presentation across species and the search for reservoir hosts. *bioRxiv* 2020:2020.06.05.135699.
134. Erlanson DA, McDowell RS, O'Brien T. Fragment-based drug discovery. *J Med Chem* 2004;47:3463–3482.
135. Hajduk PJ, Greer J. A decade of fragment-based drug design: strategic advances and lessons learned. *Nat Rev Drug Discov* 2007;6:211–219.
136. Perryman AL, Zhang Q, Soutter HH, Rosenfeld R, McRee DE et al. Fragment-based screen against HIV protease. *Chem Biol Drug Des* 2010;75:257–268.
137. Rees DC, Congreve M, Murray CW, Carr R. Fragment-based lead discovery. *Nat Rev Drug Discov* 2004;3:660–672.
138. Saalau-Bethell SM, Woodhead AJ, Chessari G, Carr MG, Coyle J et al. Discovery of an allosteric mechanism for the regulation of HCV NS3 protein function. *Nat Chem Biol* 2012;8:920–925.
139. Su H, Yao S, Zhao W, Li M, Liu J et al. Discovery of baicalin and baicalein as novel, natural product inhibitors of SARS-CoV-2 3CL protease in vitro. *bioRxiv* 2020:2020.04.13.038687.
140. Lee C-C, Kuo C-J, Ko T-P, Hsu M-F, Tsui Y-C et al. Structural basis of inhibition specificities of 3C and 3C-like proteases by zinc-coordinating and peptidomimetic compounds. *J Biol Chem* 2009;284:7646–7655.
141. Wang F, Chen C, Tan W, Yang K, Yang H. Structure of main protease from human coronavirus NL63: insights for wide spectrum Anti-Coronavirus drug design. *Sci Rep* 2016;6.
142. Zhao Q, Li S, Xue F, Zou Y, Chen C et al. Structure of the main protease from a global infectious human coronavirus, HCoV-HKU1. *J Virol* 2008;82:8647–8655.
143. Berry M, Fielding B, Gamielien J. Human coronavirus OC43 3CL protease and the potential of ML188 as a broad-spectrum lead compound: homology modelling and molecular dynamic studies. *BMC Struct Biol*. 2015;Apr 28;15:8.
144. Deng X, StJohn SE, Osswald HL, O'Brien A, Banach BS et al. Coronaviruses resistant to a 3C-like protease inhibitor are attenuated for replication and pathogenesis, revealing a low genetic barrier but high fitness cost of resistance. *J Virol* 2014;88:11886–11898.
145. Kumar V, Shin JS, Shie J-J, Ku KB, Kim C et al. Identification and evaluation of potent middle East respiratory syndrome coronavirus (MERS-CoV) 3CL pro inhibitors. *Antiviral Res* 2017;141:101–106.
146. Yin W, Mao C, Luan X, Shen D-D, Shen Q et al. Structural basis for inhibition of the RNA-dependent RNA polymerase from SARS-CoV-2 by remdesivir. *Science* 2020;368:1499–1504.

Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as 'excellent' or 'very good'.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.