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SARS-CoV-2 Papain-Like Protease: Structure, Function and Inhibition

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Emerging variants of SARS-CoV-2 and potential novel epidemic coronaviruses underline the importance of investigating various viral proteins as potential drug targets. The papain-like protease of coronaviruses has been less explored than other viral proteins; however, its substantive role in viral replication and impact on the host immune response make it a suitable target to study. This review article focuses on the structure and function of the papain-like protease (PL^{pro}) of SARS-CoV-2, including variants of concern, and compares it to those of other coronaviruses, such as SARS-CoV-1 and MERS-CoV. The protease's recognition motif is mirrored in ubiquitin and ISG15, which are involved in the antiviral immune response. Inhibitors, including GRL0617 derivatives, and their prospects as potential future antiviral agents are also discussed.

1. Introduction

The discovery of SARS-CoV-1 in 2003 induced a paradigm-shift in the assessment of coronaviruses, as it related a human coronavirus to severe illness for the first time.[1] Since the emergence of the closely related SARS-CoV-2 and COVID-19 in late 2019,[2] extraordinary efforts in pharmaceutical research have led to the rapid development of preventive vaccines (mRNA, vector, protein),[3] neutralizing monoclonal antibodies (mAbs),[4] and small molecule drugs.[5] Yet the advent of SARS-CoV-2 as the third severe coronavirus outbreak during the 21st century is a warning that future zoonotic transmissions of emerging coronaviruses are possible.[6]

SARS-CoV-1, MERS-CoV, and SARS-CoV-2 are part of the *Betacoronavirus* genus,[7] and feature a large positive-sense, single-stranded RNA genome of circa 30 kb, which encodes for accessory factors, structural proteins, and non-structural proteins.[8] The open reading frame ORF1ab spans more than two thirds of the total genome and encodes for two polyproteins (pp1a, pp1ab)[7,9] containing several non-structural proteins, many of which are essential for viral replication (Figure 1a).[10] Proteolytical processing to liberate the individual non-structural proteins is realized by the main protease (M^{pro}, 3CL^{pro}, nsp5) and the papain-like protease (PL^{pro}, part of nsp3; Figure 1b).[7,9,11]

Given that viral protease inhibitors are approved drugs against HIV and HCV,[12] both viral proteases of SARS-CoV-2 are prime drug targets. While the M^{pro} inhibitor nirmatrelvir is already approved and broadly used,[13] PL^{pro} attracted considerably less attention. However, the release of nsp1–nsp4 is likewise important for the viral replication.[7,14] Inhibition of the proteolytic activity of PL^{pro} may therefore stop the viral replication cycle.[7,14] Additional functions of PL^{pro} were identified, such as interactions with the host immune system, e.g., *via* deubiquitination and de-ISGylation.[15] In comparison to host deubiquitinases (DUBs), PL^{pro} has a slightly different substrate scope, potentially permitting the design of specific antivirals.[16]

2. Structure and Function

2.1. SARS-CoV-2 PL^{pro} is structurally conserved

PL^{pro} is one of the eight domains of the non-structural protein 3 (nsp3),[10b,14] which is the largest of the SARS-CoV-2 non-structural proteins (212 kDa).[17] The functions of the nsp3 domains within the viral replication cycle are diverse[18] and have been reviewed for SARS-CoV-1.[14] PL^{pro} is located on residues 746–1060 of nsp3 (1564–1878 of pp1ab[11b,16]; SARS-CoV-2 reference sequence WIV04)[19] and consists of 315 amino acids (36 kDa).[11b,16] PL^{pro} is highly conserved among coronaviruses. The amino acid sequence of SARS-CoV-2 PL^{pro} is 98% identical to the related proteases from bat coronaviruses BANAL-52 and RaTG13,[20] and 83% identical to the related human coronavirus SARS-CoV-1.[21] The active sites of SARS-CoV-1 and SARS-CoV-2 PL^{pro} are almost identical.[22] In contrast, the amino acid sequence of SARS-CoV-2 PL^{pro} is only 29% identical and only 51% similar to MERS-CoV PL^{pro} (Figure 2).[23] However, superimposition of X-ray crystal structures of PL^{pro} from SARS-CoV-2, SARS-CoV-1 and MERS-CoV demonstrate their structural similarity and high conservation (RMSD ≤ 1.11 Å; Figure 3a).[24] Active-site inhibitors designed for SARS-CoV-1 and SARS-CoV-2 PL^{pro} appear to display lower activity against MERS-CoV PL^{pro}, potentially challenging the discovery of pan-coronaviral PL^{pro} inhibitors.[25]

SARS-CoV-2 PL^{pro} is a cysteine protease consisting of four domains, three of which form the catalytically relevant right-handed thumb-palm-fingers architecture (Figure 3b). The catalytic triad is located between the thumb (62–178) and palm (241–315) domains, and consists of Cys111, His272, and Asp286 (Figure 3c). The fingers domain (179–240) contains a zinc binding site made up of four cysteine residues (Cys189, Cys192, Cys224, Cys226). Mutagenesis studies of these cysteine residues in SARS-CoV-1 PL^{pro} demonstrated that coordination of zinc is required for catalytic activity.[29] The fourth domain of SARS-CoV-2 PL^{pro} is a ubiquitin-like (Ub-like) domain (1–61); however, its specific function is not yet fully understood (Figure 3b).[22,30]

The general architecture of PL^{pro} resembles[14,16] that of human deubiquitinating enzymes (DUB) (e.g., ubiquitin-specific proteases; USP).[32] However, their sequences and structures appear to be dissimilar enough to allow for the development of specific PL^{pro} inhibitors, despite concerns about potential off-target effects of PL^{pro} inhibitors.[25,33] It is good practice to

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counter-screen PL[pro] inhibitors against a set of human DUBs, and in case of covalent inhibitors additionally against other human cysteine proteases.[25] A factor associated with selective binding to PL[pro] over DUBs is the highly mobile β-loop (BL2, 266–271),[14,24a,25,33] which is identical in sequence for SARS-CoV-1 and SARS-CoV-2 (Figure 2).

Since SARS-CoV-2 continues to evolve, several variants of the virus have been identified, with some classified by health authorities as variants of concern (VOC; Alpha, Beta, Gamma, Delta, Omicron).[34] These variants show pronounced genetic variability in comparison to the ancestral SARS-CoV-2 strain.[35] Mutations have accumulated particularly in the spike protein,[36] which governs host cell recognition and infectivity.[35b,37] Other viral proteins are also affected by mutations of the genome, as exemplified by the presence of certain M[pro] mutants in SARS-CoV-2 variants (e.g., Omicron M[pro] P132H).[38] An analysis of the SARS-CoV-2 VOC genomes with the Outbreak.info database[39] revealed the presence of three abundant (> 20%) point mutations in PL[pro]. While the Delta and Omicron variants do not display a change in the amino acid sequence of PL[pro], the Alpha (Ala145Asp, > 95%), Beta (Lys92Asn, > 90%) and Gamma (Lys232Gln, > 95%) VOCs contain single amino acid mutations in PL[pro] (Figure 3d). The Lys232Gln mutation present in SARS-CoV-2 Gamma is located in the fingers domain of PL[pro] and was associated with enhanced catalytic activity in vitro.[40] Notably, the mutations Tyr268Thr (as found in MERS-CoV PL[pro]) and Tyr268Gly impact negatively on the binding of naphthalene-based inhibitors such as GRL0617.[17]

2.2. SARS-CoV-2 PL[pro] processes the viral polyproteins

One function of PL[pro] is the liberation of the first non-structural proteins (nsp1–nsp4), which are essential for viral replication (Figure 1b).[7] By binding to the human ribosome, nsp1 inhibits host cell translation,[41] and nsp2 is thought to disrupt the cell cycle progression and apoptosis.[42] Apart from PL[pro], nsp3 contains seven other domains and is among other functions involved in facilitating the assembly of the replication and transcription complex (RTC),[10b,14,42] as is nsp4.[42] Accordingly, SARS-CoV-2 PL[pro] is involved in its autocleavage,[43] equivalent to the second viral protease M[pro].[11a] It may be worth noting that nsp3 of certain coronaviruses contain an additional PL[pro], as is

the case for the human coronaviruses NL63, OC43, HKU1, and 229E.[44]

PL[pro] recognizes Leu-Xaa-Gly-Gly↓Xaa substrate motifs (P$_4$–P$_1'$, Schechter-Berger nomenclature)[45] in the viral polyprotein (pp1a, pp1ab).[46] The PL[pro] cleavage sites (P$_5$–P$_5'$) within the viral polyprotein are identical between SARS-CoV-2 and its closest known bat coronavirus relatives RaTG13 and BANAL-52 (Table 1). Similarly, the PL[pro] cleavage sites (P$_5$–P$_5'$) of SARS-CoV-2, SARS-CoV-1 and MERS-CoV polyproteins share a high degree of identity (Table 1, Figure 4). The requirement for Gly in P$_1$ and P$_2$ was confirmed by a combinatorial substrate library study of SARS-CoV-1 and SARS-CoV-2 PL[pro].[46] The protease's recognition is less specific in P$_3$ and P$_4$, as demonstrated by substrates containing homotyrosine (hTyr; P$_3$) and diaminopropionic acid (Dap; P$_4$).[46]

2.3. SARS-CoV-2 PL[pro] acts as deubiquitinase

SARS-CoV-2 PL[pro] participates in the virus's suppression of the host immune system.[11b] Its effect on specific immunological pathways was comprehensively reviewed by Mahmoudvand et al.[11b] Importantly, PL[pro] removes post-translational ubiquitination and ISG-ylation,[43] which are central protein modifications of the antiviral immune response.[48] In vitro studies showed that SARS-CoV-2 PL[pro] catalyzes the hydrolysis of fluorogenic Ub and ISG15 (interferon-stimulated gene 15) substrates.[46] PL[pro] recognizes the C-terminus of ubiquitin (Leu73-Arg74-Gly75-Gly76).[46] Similarly, it was shown that the six C-terminal residues of ISG15 (Leu152-Arg153-Leu154-Arg155-Gly156-Gly157) are essential for its interaction with PL[pro].[49] Evidently, the C-terminal amino acid sequences of Ub and ISG15 are related to the protease's viral substrate recognition sequences (Table 1).[46,49b]

2.4. SARS-CoV-2 PL[pro] interacts with ubiquitin-like proteins

Ub and ISG15 interact with two binding sites (S1, S2) on the surface of SARS-CoV-2 PL[pro] (Figure 5).[16,50] Mono-Ub binds to the S1 site of the enzyme (Figure 5a), while Lys48-linked poly-Ub interacts with both the S1 and S2 sites (Figure 5c).[16] While SARS-CoV-2 PL[pro] can bind mono-Ub, poly-Ub is preferentially

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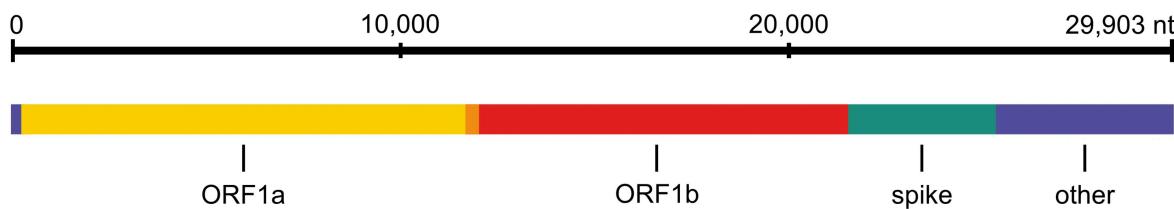
SARS-CoV-2 genome

b

ORF1ab polyproteins
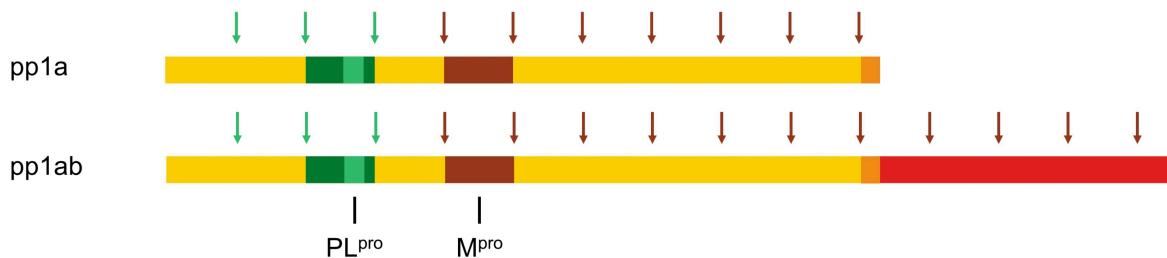
Figure 1. Genome and polyprotein organization of SARS-CoV-2. (a) SARS-CoV-2 RNA genome with representations of the genes of ORF1a (yellow, orange), ORF1b (orange, red), and the spike (teal). (b) SARS-CoV-2 ORF1ab polyproteins pp1a and pp1ab. The arrows indicate a cleavage site recognized by either PL[pro] (green) or M[pro] (brown). Individual sections of the polyproteins are not to scale.

Table 1. PL[pro] cleavage sites of different coronaviruses.[a]

	nsp1↓nsp2	nsp2↓nsp3	nsp3↓nsp4
SARS-CoV-2	ELNGG↓AYTRY	TLKGG↓APTKV	ALKGG↓KIVNN
SARS-CoV	ELNGG↓AVTRY	RLKGG↓APIKG	SLKGG↓KIVST
MERS-CoV	KLIGG↓DVTPV	RLKGG↓APVKK	KIVGG↓APTWF
BANAL-52	ELNGG↓AYTRY	TLKGG↓APTKV	ALKGG↓KIVNN
RaTG13	ELNGG↓AYTRY	TLKGG↓APTKV	ALKGG↓KIVNN

[a] Substrate recognition sequence is shown from P_5–P_5' according to the Schechter-Berger nomenclature[45] within the viral polyprotein (pp1a, pp1ab) between nsp1, nsp2, nsp3, and nsp4 of SARS-CoV-2,[19] SARS-CoV-1,[21] MERS-CoV,[23] BANAL-52,[20a] and RaTG13.[20b] The arrow (↓) indicates the position of proteolysis within the sequence. The consensus sequence over the three cleavage sites is illustrated in Figure 4.

processed.[16] Notably, Lys48-linked poly-Ub substrates are cleaved less efficiently by SARS-CoV-2 PL[pro] than by SARS-CoV-1 PL[pro], which may be due to the cumulative effect of amino acid substitutions in the S2 binding site.[40,46,50] ISG15 consists of two Ub-like domains linked by a short hinge region.[48c] When interacting with PL[pro], ISG15 binds simultaneously to S1 and S2. The C-terminal domain (ISG15[CTD]) binds to S1 while the N-terminal domain (ISG[NTD]) binds to S2 (Figure 5b, Figure 5d). While ISG15 and Ub interact with PL[pro] at the same surfaces, there are specific dissimilarities in their binding geometry (see Figure 5a vs. Figure 5b), which were thoroughly reviewed by Olsen and co-workers.[50] As most host DUBs preferentially bind mono-Ub, the slightly different substrate scope of PL[pro] may be beneficial for the development of less cross-reactive, specific inhibitors.[16]

3. PL[pro] Inhibitors

Several inhibitors of SARS-CoV-2 PL[pro] were originally identified as inhibitors of SARS-CoV-1 PL[pro]. Among those is GRL0617, which was discovered by Ratia et al.,[52a] and has since become one of the most frequently studied inhibitors of SARS-CoV-2 PL[pro] (Figure 6).[16,24a,49b,52b,53] This small molecule is a reversible competitive inhibitor of PL[pro] (Figure 7a, Figure 7b).[52a] Half-maximal inhibitory concentrations (IC_{50}) of GRL0617 against SARS-CoV-2 PL[pro] have been determined in various biochemical *in vitro* assays and range from 0.88 μM to 2.6 μM.[52b,c] Evaluations of the compound in a cell culture-based assay indicated micromolar half-maximal effective concentrations ($EC_{50} = 20$ μM–33 μM).[52b,53c] GRL0617 is ineffective against MERS-CoV PL[pro] due to a single amino acid substitution – an effect which could similarly be observed in recombinant SARS-CoV-2 PL[pro] mutants (Tyr268Thr, Tyr268Gly).[17]

Two small molecules which resulted from the same screening campaign against SARS-CoV-1 conducted by Ratia et al.[52a] were also tested against SARS-CoV-2 PL[pro] and showed high micromolar inhibition (7724772, $IC_{50} = 24$ μM; 6577871, $IC_{50} = 101$ μM).[53a] Three further SARS-CoV-1 PL[pro] inhibitors were assessed against SARS-CoV-2 PL[pro] by Klemm et al.[16] The

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                  βA                   βB        TT    αA      βC   βD         ηA
              1        10          20              30        40         50
SARS-CoV-2   .EVRTIKVFTTVDNINLHTQVVDMSMTYGQQFGPTYLDGADVTKIKPHNSHEGK
SARS-CoV-1   REVKTIKVFTTVDNTNLHTQLVDMSMTYGQQFGPTYLDGADVTKIKPHVNHEGK
MERS-CoV     ..QLTIEVLVTVDGVNFRTVVLNNKNTYRSQLGCVFFNGADISDTIPDEKQNGH
BANAL-52     .EVRTIKVFTTVDNINLHTQVVDMSMTYGQQFGPTYLDGADVTKIKPHNSHEGK
RaTG13       .EVRTIKVFTTVDNINLHTQVVDMSMTYGQQFGPTYLDGADVTKIKPHNSHEGK

              βE        αB                   αC            βF  βG
                    60          70        80          90      100
SARS-CoV-2   TFYVLPNDDTLRV.EAFEYYHTTDPSFLGRYMSALNHTKKWKYPQVNGLTSIKW
SARS-CoV-1   TFFVLPSDDTLRS.EAFEYYHTLDESFLGRYMSALNHTKKWKFPQVGGLTSIKW
MERS-CoV     SLYLADNLTADETKALKELYGPVDPTFLHRFYSLKAAVHGWKMVVCDKVRSLKL
BANAL-52     TFYVLPNDDTLRA.EAFEYYHTTDPSFLGRYMSALNHTKKWKYPQVNGLTSIKW
RaTG13       TFYVLPNDDTLRA.EAFEYYHTTDPSFLGRYMSALNHTKKWKYPQVNGLTSIKW

                  αD                    αE              αF            T
              110        120        130        140        150
SARS-CoV-2   ADNNCYLATALLTLQQI.ELKFNPPALQDAYYRARAGEAANFCALILAYCNKTV
SARS-CoV-1   ADNNCYLSSVLLALQQL.EVKFNAPALQEAYYRARAGDAANFCALILAYSNKTV
MERS-CoV     SDNNCYLNAVIMTLDLLKDIKFVIPALQHAFMKHKGGDSTDFIALIMAYGNCTF
BANAL-52     ADNNCYLATALLTLQQI.ELKFNPPALQDAYYRARAGEAANFCALILAYCNKTV
RaTG13       ADNNCYLATALLTLQQI.ELKFNPPALQDAYYRARAGEAANFCALILAYCNKTV

              T     αG          T.T    βH    TTT    βI    αH  βJ
              160        170        180        190        200        210
SARS-CoV-2   GELGDVRETMSYLFQHANLD.SCKRVLNVVCKTCGQQTTLKGVEAVMYMGTLS
SARS-CoV-1   GELGDVRETMTHLLQHANLE.SAKRVLNVVCKHCGQKTTTLTGVEAVMYMGTLS
MERS-CoV     GAPDDASRLLHTVLAKAELCCSARMVWREWCNVCGIKDVVLQGLKACCYVGVQT
BANAL-52     GELGDVRETMNHLFQHANLD.SCKRVLNVVCKTCGQQTTLKGVEAVMYMGTLS
RaTG13       GELGDVRETMNYLFQHANLD.SCKRVLNVVCKTCGQQTTLKGVEAVMYMGTLS

              αI      βK       TT       βL              βM          TT       βN
                    220        230        240        250        260
SARS-CoV-2   YEQFKKGVQIPCTCGKQATKYLVQQESPFVMMSAPPAQYELKHGT...FTCASE
SARS-CoV-1   YDNLKTGVSIPCVCGRDATQYLVQQESSFVMMSAPPAEYKLQQGT...FLCANE
MERS-CoV     VEDLRARMTYVCQCGGERHRQLVEHTTPWLLLSGTPNEKLVTTSTAPDFVAFNV
BANAL-52     YEQLKKGVQIPCTCGKQATQYLVQQESPFVMMSAPPAQYELKHGT...FICASE
RaTG13       YEQLKKGVQIPCTCGKQATQYLVQQESPFVMMSAPPAQYELKHGT...FICASE

                      βO            βP     TT  βQ        βR              βS
                    270        280        290        300        310
SARS-CoV-2   YTG.NYQCGHYKHITSKET.LYCIDGALLTKSSEYKGPITDVFYKENSYTTTIK
SARS-CoV-1   YTG.NYQCGHYTHITAKET.LYRIDGAHLTKMSEYKGPVTDVFYKETSYTTTIK
MERS-CoV     FQGIETAVGHYVHARLKGGLILKFDSGTVSKTSDWKCKVTDVLFPGQKYSSD..
BANAL-52     YTG.NYQCGHYKHITSKET.LYCIDGALLTKSSEYKGPITDVFYKENSYTTTIK
RaTG13       YTG.NYQCGHYKHITSKET.LYCIDGALLTKSSEYKGPITDIFYKENSYITTIK
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Figure 2. Alignment of the amino acid sequences of PL[pro]. Sequences originate from SARS-CoV-2 (WIV04)[19] and related coronaviruses (% identity, % similarity): SARS-CoV-1 (83%, 88%),[21] MERS-CoV (29%, 51%),[23] BANAL-52 (98%, 98%),[20a] RaTG13 (98%, 98%).[20b] Secondary structural elements of crystallized SARS-CoV-2 PL[pro] are indicated (PDB: 6WZU).[24a] The alignment was generated with Clustal Omega,[26] calculations were performed in Sequence Manipulation Suite 2,[27] and the illustration created in ESPript 3.[28]

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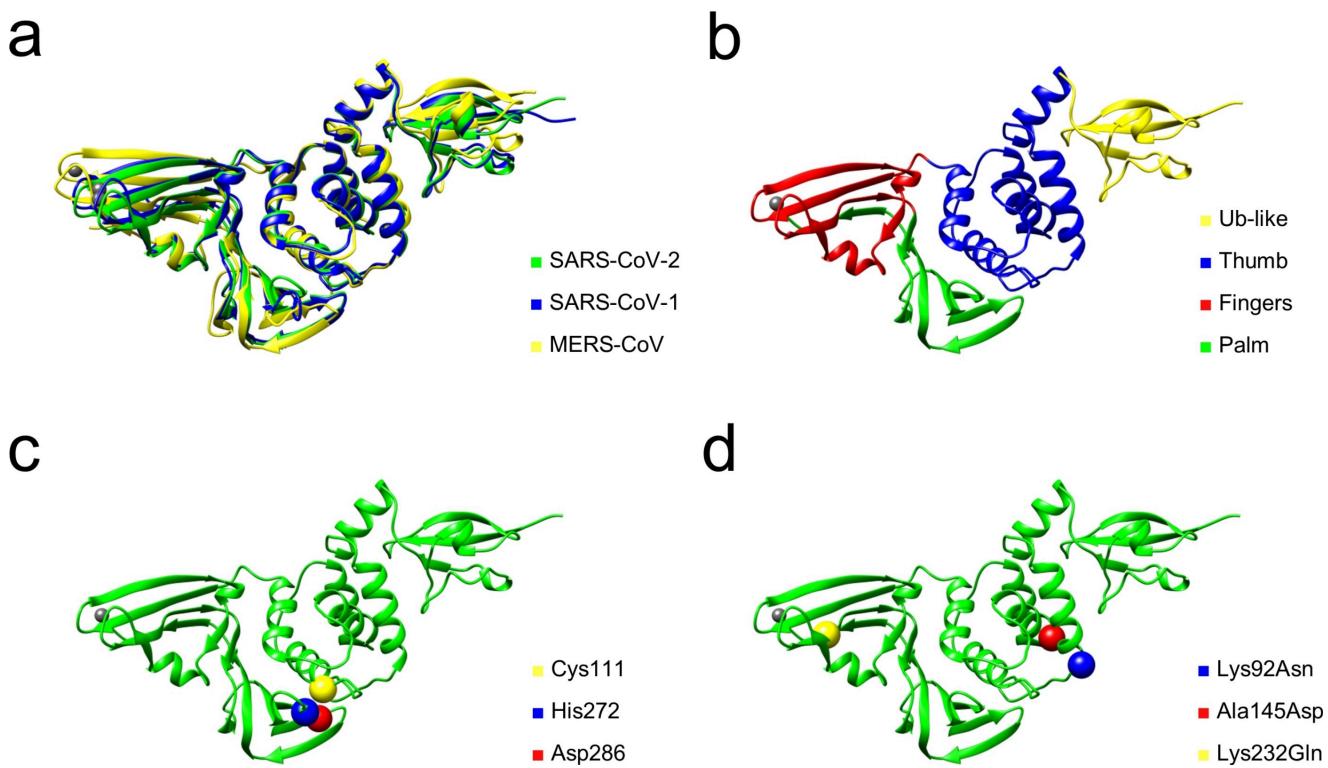
Figure 3. X-ray crystal structure representations of PL[pro]. Zinc ions are indicated in the Fingers domain as gray spheres. (a) Superimposition of PL[pro] of SARS-CoV-2 (PDB: 6WZU),[24a] SARS-CoV-1 (RMSD: 0.95 Å; PDB: 2FE8),[24b] and MERS-CoV (RMSD: 1.11 Å; PDB: 4RNA).[24c] (b) Domains of SARS-CoV-2 PL[pro]: Ub-like (1-61), thumb (62-178), fingers (179-240), palm (241-315). (c) Location of the catalytic triad of SARS-CoV-2 PL[pro]: Cys111 (yellow), His272 (blue), Asp286 (red). (d) Location of mutations of PL[pro] in SARS-CoV-2 variants of concern (VOC): Lys92Asn, Beta (blue), Ala145Asp, Alpha (red), Lys232Gln, Gamma (yellow). The graphics were generated with UCSF Chimera.[31]
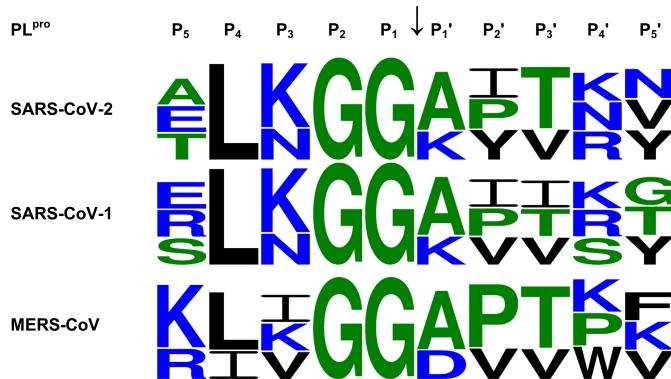
Figure 4. Viral polyprotein cleavage sites recognized by PL[pro]. Represented are the recognition motifs of SARS-CoV-2,[19] SARS-CoV-1,[21] and MERS-CoV,[23] covering residues P_5–P_5' (Schechter-Berger nomenclature)[45] within pp1a and pp1ab. Note that the recognized polyprotein sequences of SARS-CoV-2,[19] BANAL-52,[20a] and RaTG13[20b] are identical from P_5–P_5' (Table 1). The consensus sequence over the three cleavage sites (nsp1↓nsp2, nsp2↓nsp3, nsp3↓nsp4) was plotted using WebLogo 3.[47]

naphthalene-containing small molecules rac5c, rac3j, and rac3k – originally developed by Mesecar and co-workers[33] – inhibit SARS-CoV-2 PL[pro] as strongly as GRL0617 (rac5c, $IC_{50}=0.81$ μM; rac3j, $IC_{50}=1.4$ μM; rac3k, $IC_{50}=1.2$ μM).[16]

The majority of reported SARS-CoV-2 PL[pro] inhibitors are repurposed small molecules identified from high-throughput

screenings. A study by Lim et al. identified five small molecules with sub-micromolar *in vitro* activity against SARS-CoV-2 PL[pro]: GNF-Pf-3800 ($IC_{50}=0.26$ μM), ryuvidine ($IC_{50}=0.39$ μM), Ro 08–2750 ($IC_{50}=0.53$ μM), dihydrotanshinone I ($IC_{50}=0.59$ μM), and beta-lapachone ($IC_{50}=0.61$ μM).[53c] The same study also found tanshinone IIA ($IC_{50}=1.6$ μM) and cryptotanshinone ($IC_{50}=1.3$ μM) to be single-digit micromolar inhibitors of SARS-CoV-2 PL[pro],[53c] and the latter was also identified in a high-throughput screening performed by Zhao et al. ($IC_{50}=5.6$ μM).[53d] This screening also identified tanshinone I ($IC_{50}=2.2$ μM) and YM155 ($IC_{50}=2.5$ μM) as SARS-CoV-2 PL[pro] inhibitors.[53d] Lim et al. also reported that Ro 08–2750 ($EC_{50}=20$ μM) and dihydrotanshinone I ($EC_{50}=8.1$ μM) were active against SARS-CoV-2 in a cell culture-based assay.[53c] On the contrary, Wang and Ma reported that tanshinone I, tanshinone IIa, cryptotanshinone, and dihydrotanshinone I were inactive in both thermal-shift binding SARS-CoV-2 PL[pro] and cell culture-based anti-coronaviral assays, suggesting nonspecific interactions.[54]

Additional SARS-CoV-2 PL[pro] inhibitors were discovered in another high-throughput screening by Wang and co-workers.[52b] Extensive structure-activity-relationship (SAR) studies on the basis of two micromolar hits led to the discovery of 30 related small molecule inhibitors of SARS-CoV-2 PL[pro] (e.g., Jun9-75-5, $IC_{50}=0.56$ μM).[52b] Multiple compounds were also evaluated in cell-based antiviral assays, in which Jun9-72-2
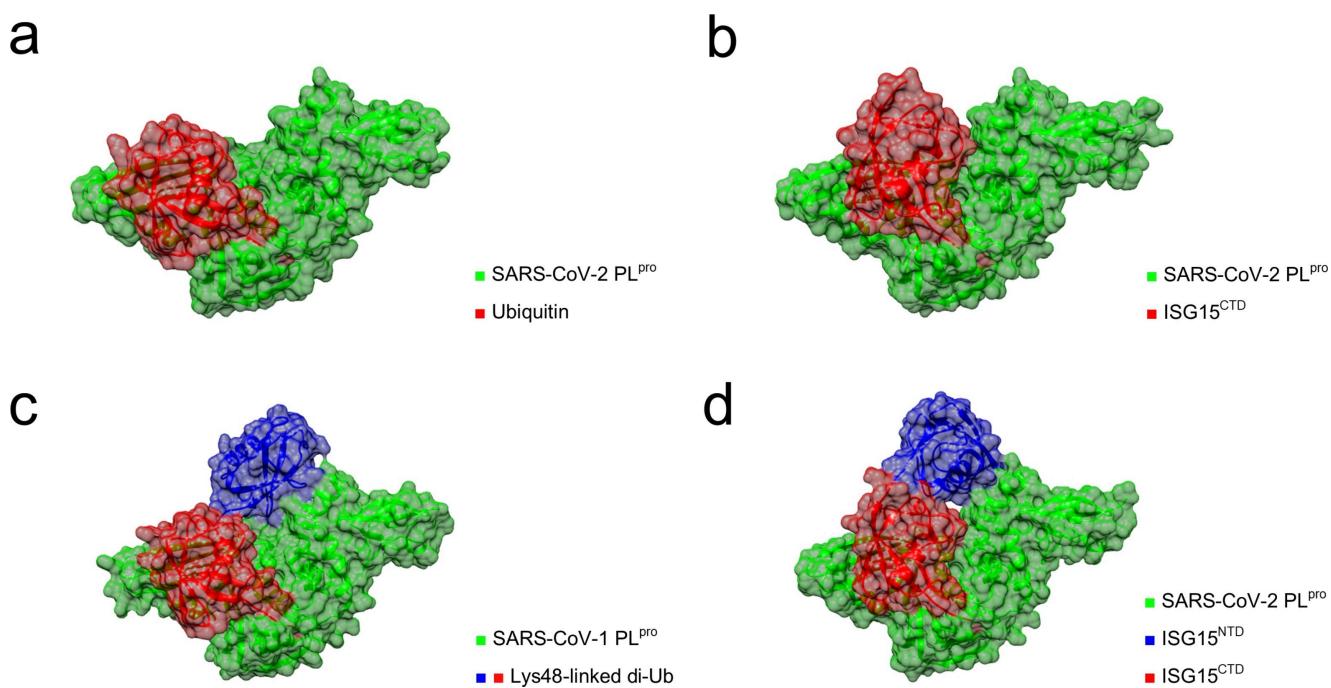
Figure 5. X-ray crystal structures of PL^{pro} in complex with Ub-like proteins. (a) SARS-CoV-2 PL^{pro} in complex with ubiquitin bound to the S1 binding site (PDB: 6XAA)[16] (b) SARS-CoV-2 PL^{pro} in complex with ISG15^{CTD} (PDB: 6XA9).[16] (c) SARS-CoV-1 PL^{pro} in complex with a Lys48-linked di-ubiquitin probe bound to the S1 and S2 binding sites (PBD: 5E6J).[51] (d) SARS-CoV-2 PL^{pro} in complex with ISG15 (PDB: 7RBS).[24a] The graphics were generated with UCSF Chimera.[31]
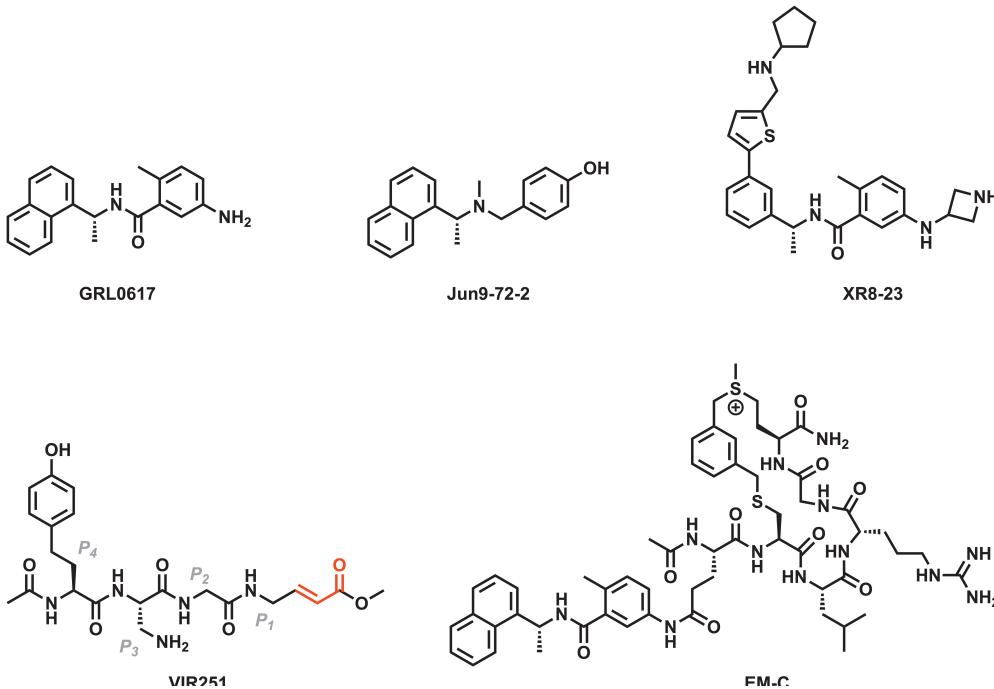

Figure 6. Chemical structures of selected inhibitors of PL^{pro}.[33,46,52] The α,β-unsaturated carbonyl of VIR251 is relevant for the compound's Michael reactivity with the catalytic cysteine (Cys111) and is highlighted in red. Binding subsites of VIR251 are indicated.

(Figure 6) displayed the strongest anti-coronaviral activity (EC$_{50}$ = 6.6–7.9 μM).[52b]

Structure-based efforts to improve activity led to the discovery of several sub-micromolar SARS-CoV-2 PL^{pro} inhibitors.[55] Central to the study was the replacement of the common naphthalene motif by a 2-phenylthiophene moiety.[55] Inhibitors like XR8-23 (IC$_{50}$ = 0.39 μM, EC$_{50}$ = 1.4 μM, Figure 6) interact with the protease through a novel binding mode by

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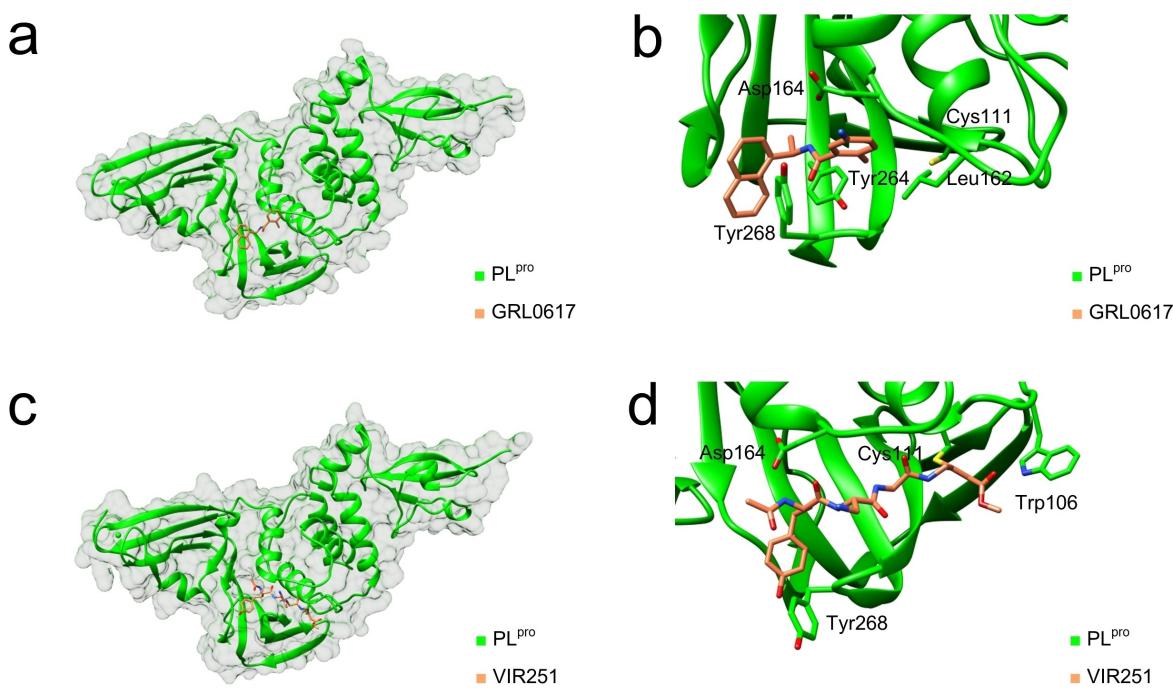
Figure 7. X-ray co-crystal structures of inhibitor-bound SARS-CoV-2 PL[pro]. (a) PL[pro] in complex with bound GRL0617 (PDB: 7JRN).[52b] (b) Close-up view of GRL0617 (Figure 6) in the active site with key residues[49b] highlighted (PDB: 7JRN).[52b] (c) PL[pro] in complex with covalently-bound VIR251 (PDB: 6WX4).[46] (d) Close-up view of VIR251 (Figure 6) in the active site with key residues[40] highlighted (PDB: 6WX4).[46] The graphics were generated with UCSF Chimera.[31]

associating with BL2 close to the active site, and appear to have promising *in vivo* pharmacokinetics.[55]

By featuring peptide substrates of SARS-CoV-2 PL[pro] with C-terminal electrophilic vinylmethyl ester warheads, Rut et al. developed covalently binding peptidomimetics.[46] The inhibitors VIR250 and VIR251 show IC$_{50}$ values in the two-digit micromolar range against both SARS-CoV-1 and SARS-CoV-2 PL[pro] (Figure 6).[46] Patchett et al. further characterized the interactions of VIR250 and VIR251 with the active site of PL[pro] by X-ray crystallography (Figure 7c, Figure 7d).[40] Recently, several antiviral peptide-drug conjugates targeting SARS-CoV-2 PL[pro] were also reported.[52c] The peptide-drug conjugate EM-C consists of a sulfonium-tethered peptide substrate linked to GRL0617 and was reported to have an IC$_{50}$ of 7.4 μM against SARS-CoV-2 PL[pro] (Figure 6).[52c]

It was also suggested that ebselen, disulfiram and their analogues are SARS-CoV-2 PL[pro] inhibitors.[56] However, it is noted that these compounds are non-specific cysteine modifiers.[54,57]

At the time of writing, six clinical studies registered on ClinicalTrials.gov were associated with PL[pro],[58] evaluating isotretinoin-containing treatments and ebselen. The use of isotretinoin as PL[pro] inhibitor appears to be mainly supported by published *in silico* data.[59] Ebselen was also investigated as SARS-CoV-2 M[pro] inhibitor, but – as discussed previously – has been found to be an unspecific modifier of cysteine proteases.[54,57] All six studies were initially uploaded on ClinicalTrials.gov in early to mid-2020.[58] As of July 2022, the four studies involving isotretinoin-derived treatments were not

yet recruiting and the two studies on ebselen were enrolling by invitation.[58]

4. Conclusion and Outlook

PL[pro] is a promising drug target for coronavirus infections. The cysteine protease is arranged in the characteristic thumb-palm-fingers architecture and hydrolyses the viral polyproteins at three defined positions, always after Gly-Gly motifs. PL[pro] also targets posttranslational ubiquitin and ISG15 modifications, which are part of the antiviral immune response. The association of PL[pro] with both the viral replication cycle and the modulation of the host immune response is a unique opportunity for drug development.

Various SARS-CoV-2 PL[pro] inhibitors have been explored. Repurposed molecules exhibited SARS-CoV-2 PL[pro] inhibition,[53c,d] and peptide-based compounds were also explored.[46,52c] Notably, many promising inhibitors are derived from the small molecule GRL0617, which was first developed as a SARS-CoV-1 PL[pro] inhibitor.[52a] Encouraging advancements of GRL0617 include the compounds designed by Wang and co-workers,[52b] as well as the 2-phenylthiophene compounds described by Shen et al.,[55] which exhibit low micromolar activity in cell culture-based assays.[52b] However, further improvement of the pharmacokinetic and pharmacodynamic properties might be necessary to advance to clinical trials.[60]

In the case of PL[pro], small molecules with reversible binding modes were more explored than covalent peptide-based inhibitors. This is a notable difference to M[pro], where the latter

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class dominates the drug discovery landscape.[61] An obvious disadvantage of covalent inhibitors is their potential proneness to off-target effects,[62] while the additional covalent bond to the catalytically active residue boosts affinity and may offer better protection against resistance. The development of substrate-derived peptidomimetics may be challenged due to the Gly-Gly (P_2–P_1) recognition motif of PL^{pro}, as compared to the unique Gln (P_1) specificity of M^{pro}.[11a] In contrast to the rapid success with M^{pro} inhibitors like nirmatrelvir,[5b,13] the pharmacological profiles of PL^{pro} inhibitors might so far have prevented an approved drug candidate.

The COVID-19 pandemic continues to have a devastating impact on societies worldwide.[63] While the first anti-coronaviral drugs are available, the possibility of emerging resistance should not be disregarded.[64] To enhance the toolbox available in the fight against COVID-19, SARS-CoV-2 PL^{pro} appears to be a suitable target for the creation of new antivirals.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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REVIEW

Viral mutations highlight the importance of exploring a broad range of antiviral drug targets. The involvement of the SARS-CoV-2 papain-like protease in the viral replication cycle and the host immune response offers a unique possibility for drug development. In our review, we focus on the structure and function of PL^pro and discuss prospects of current inhibitors.
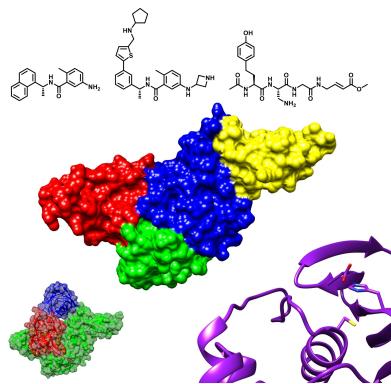
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**SARS-CoV-2 Papain-Like Protease:
Structure, Function and Inhibition**