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Proteases of SARS Coronaviruses

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Abstract

Coronaviruses such as SARS and SARS-CoV-2 have established themselves as a global health concern after causing an epidemic and a pandemic in the last twenty years. Understanding the life cycle of such viruses is critical to reveal their pathogenic potential. As one of the essential viral enzymes, SARS proteases are indispensable for the processing of viral polypeptides and for the replication of the virus. SARS-CoV and SARS-CoV-2 encode for 2 viral proteases: the main protease (3CLpro) and the papain-like protease (PLPro), which are conserved among different coronaviruses and are absent in humans. This review summarizes the existing literature on the structure and function of these proteases; highlighting the similarity and differences between the enzymes of SARS and SARS-CoV-2. It also discusses the development of inhibitors to target viral proteases.

Key Points

- The coronaviral genome encodes 2 proteases – a main protease (3CLpro) and a papain-like protease (PLPro); both are cysteine proteases. Upon infection, they cleave the viral polyprotein to form 16 non-structural proteins (Nsps).
- 3CLpro cleaves the viral polyprotein pp1a at 11 sites to form 12 active non-structural proteins (Nsps). PLpro cleaves the polyprotein at 3 sites to form 4 active Nsps. These Nsps are needed for the replication of viral ssRNA genome.
- Comparison of crystallographic structures of 3CLpro from SARS-CoV and SARS-CoV2 show subtle differences in the dimerization domain of 3CLpro.
- PLpro cleaves the viral polyprotein and host cell ubiquitin and ISG15.
- PLpro of SARS-CoV and SARS-CoV-2 are 83% identical, but still differ in terms of their substrate preference towards ubiquitin and ISG15. PLpro of SARS-CoV is a preferred deubiquitinase, while PLpro of SARS-CoV-2 is a preferential deISGylase.
- This difference in substrate specificity leads to altered regulation of host cell inflammatory signaling and type I interferon response.
- High throughput assays are important to test protease activity in drug screens to discover novel inhibitors of viral proteases.

Introduction

Coronaviruses are a Major Health Concern

Coronaviruses (CoVs) have recently emerged as a global health concern, causing two epidemics and a pandemic in only 20 years. The effect of CoVs on human health began with the epidemic of severe acute respiratory syndrome (SARS) in 2002–2003. In 2012, the Middle East respiratory syndrome (MERS) was a severe health threat in 27 different countries, but ~80% cases were confined to Saudi Arabia. At the end of December 2019, another CoV outbreak emerged, which was declared as the COVID-19 pandemic by the World Health Organization. SARS and MERS had higher mortality, but lower transmission rates when compared to SARS-CoV-2 (see "Relevant Websites" section).

Origin of Pathogenic Coronaviruses

Coronaviruses are enveloped viruses with a positive-sense, single-stranded RNA genome, which is approximately 30 kb in size. They belong to the coronaviridae family. Based on their antigenic and genetic properties, CoVs are organized into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. There are seven coronaviruses that infect humans. Four of them (229E, NL63, OC43, HKU1) are common human coronaviruses and cause a mild illness, while three others (SARS, MERS, SARS-CoV-2) caused severe acute respiratory syndromes (Liu *et al.*, 2020a). In the latter three cases, the human disease was caused by interspecies transmission of zoonotic RNA viruses. The viruses are believed to have originated in bats, and then transferred to humans through intermediate hosts, such as market civets (in case of SARS) and dromedary camels (in case of MERS) (V'kovski *et al.*, 2021).

The Coronaviral Genome

The first portion of the coronaviral genome encodes ORF1a/b, which produces 2 polypeptides pp1a and pp1ab after viral infection. These are cleaved by viral proteases to produce 16 non-structural proteins (Nsps). There are 2 coronaviral viral proteases: the chymotrypsin-like protease (3CLpro; also called the main protease) and one or two papain-like proteases (PLpro). The nsps encoded by ORF1a/b include the viral proteases, the RNA-dependent RNA polymerase, helicase and mRNA capping enzymes. The next part of the genome encodes subgenomic RNAs (sgRNAs) that are translated after viral replication. The sgRNAs encode the structural and accessory proteins of CoVs. The four main structural proteins are spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. Accessory proteins are needed for genome maintenance and viral replication (V'kovski *et al.*, 2021).

The Viral Lifecycle

The attachment of the virion to the host cell requires interactions between the S protein and its receptor. Both SARS-CoV and SARS-CoV-2 use angiotensin-converting enzyme 2 (ACE2) as their receptor, while MERS-CoV binds to dipeptidyl-peptidase 4 (DPP4) to allow entry into human cells. The S protein is then cleaved by the host protease TMPRRS2, which causes fusion of the viral and cellular membranes. This releases the viral genome into the host cytoplasm. The virus then utilizes ribosomal frameshifting to translate the polyproteins pp1a and pp1ab from ORF1a/b. These polyproteins are subsequently cleaved into the individual Nsps by the viral PLpro, and 3CLpro. The Nsps assemble to form an active replicase–transcriptase complex (RTC), which facilitates RNA replication and transcription of the sub-genomic RNAs. The viral structural proteins, S, E, and M are then translated from the sgRNAs and inserted into the endoplasmic reticulum (ER). These proteins move along the secretory pathway into the endoplasmic reticulum–Golgi intermediate

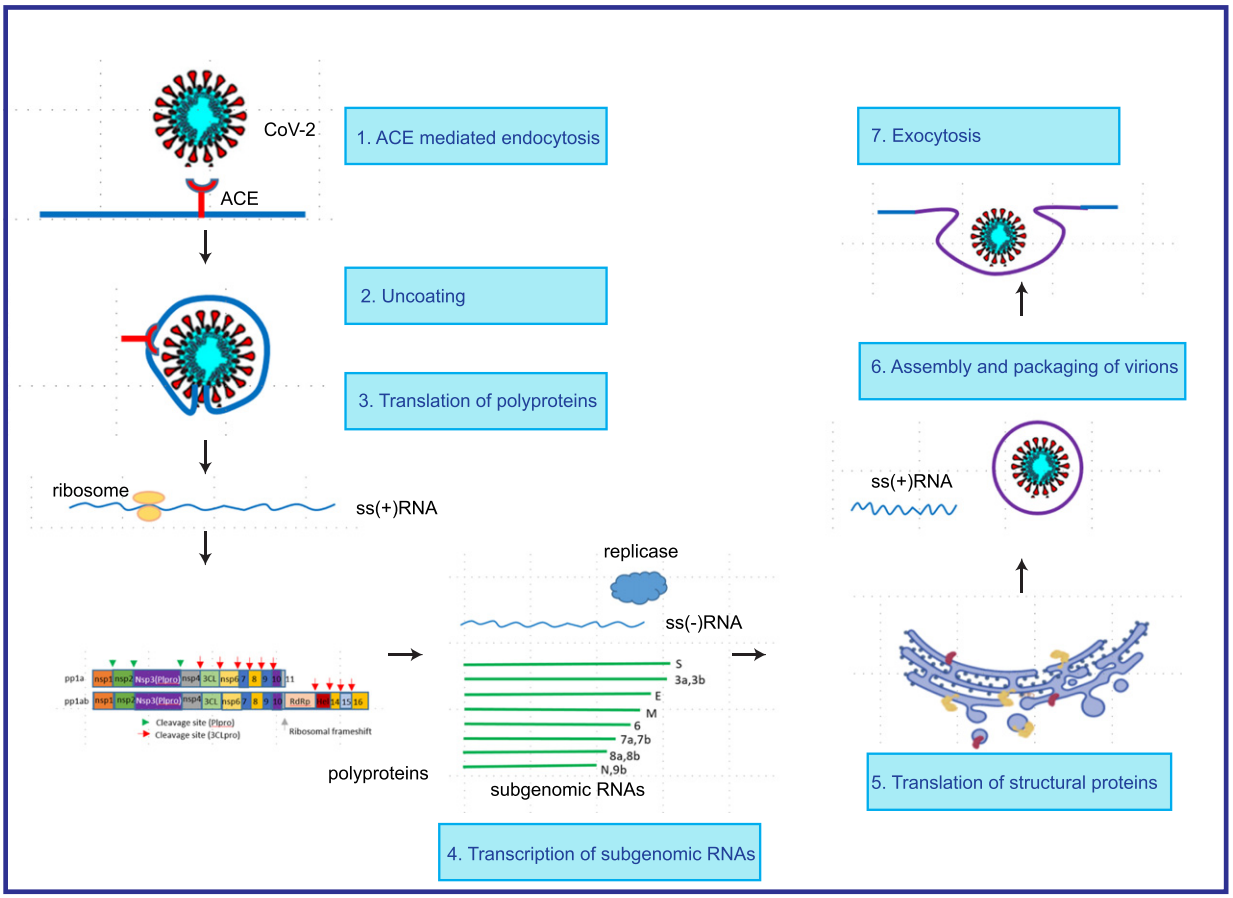

Fig. 1 Lifecycle of SARS-CoV-2. The virus enters the cell using ACE2 receptor mediated endocytosis. This is followed by uncoating which releases the ss(+) RNAgenome, followed by translation of polyproteins which are then processed by 3CL-Pro and PLpro to generate the active replicase and other viral proteins. The active replicase then helps in formation of ss(-)RNA and transcription of subgenomic RNAs which encode structural proteins needed for virion assembly. Assembly and packaging of virions is followed by exocytosis of viral progeny from the cell.

compartment (ERGIC). Replicated viral RNA is encapsulated by the N protein and assembled into mature virions at the ERGIC. Mature virions are then released from the cell by utilizing lysosomal exocytosis (V'kovski *et al.*, 2021; Stukalov *et al.*, 2021) (**Fig. 1**).

This review is focused on coronaviral proteases and their role in infection. Both the viral main protease (3CLpro) and papain-like protease (PLpro) are essential for viral replication and maturation (PLpro). In addition, the papain-like protease also modulates host cell metabolism through its regulation of ubiquitin and ISG15 dependent pathways. Therefore, understanding the structure and functions of the proteases is critical for developing specific antiviral drugs that can effectively prevent or treat coronaviral diseases. SARS-CoV-2 shares a higher (79%) genetic similarity with SARS-CoV than it does with MERS-CoV (50%). Though the genetic similarity is high, SARS-COV and SARS-CoV-2 have significant differences in terms of molecular details, epidemiology and clinical symptoms. We have focused this review on understanding the similarities and differences between the proteases of SARS-CoV and SARS-CoV-2, which may help explain some of the differences in the disease pathogenesis and host immune response in SARS and COVID-19.

The Main Protease (MPro or 3CLpro)

The main protease of coronaviruses is encoded by the nsp5 gene. Upon infection, 3CLpro cleaves pp1a at 11 conserved sites to generate 12 active non-structural proteins (Nsp4-Nsp16), which are needed for replication and synthesis of viral subgenomic RNAs. From the N to the C terminus, the amino acids in the substrates are numbered as -P4-P3-P2-P1↓P1′-P2′-P3′-), and the cleavage site is located between P1 and P1′. A Gln residue is almost always required in the P1 position of the substrates. The protease cleavage site often resembles the sequence of (Leu,Met,Phe)-Gln-(Ser,Ala,Gly), where the proteolytic cleavage occurs at the C-terminal of Gln (Liu *et al.*, 2020a,b). The main protease is indispensable for viral replication. It is also highly conserved among different coronaviruses, such as SARS-CoV, MERS-CoV, TGEV, HCoV 229E and SARS-CoV-2, but is absent in humans, making it a promising target for anti-coronaviral drugs (Ma *et al.*, 2020).

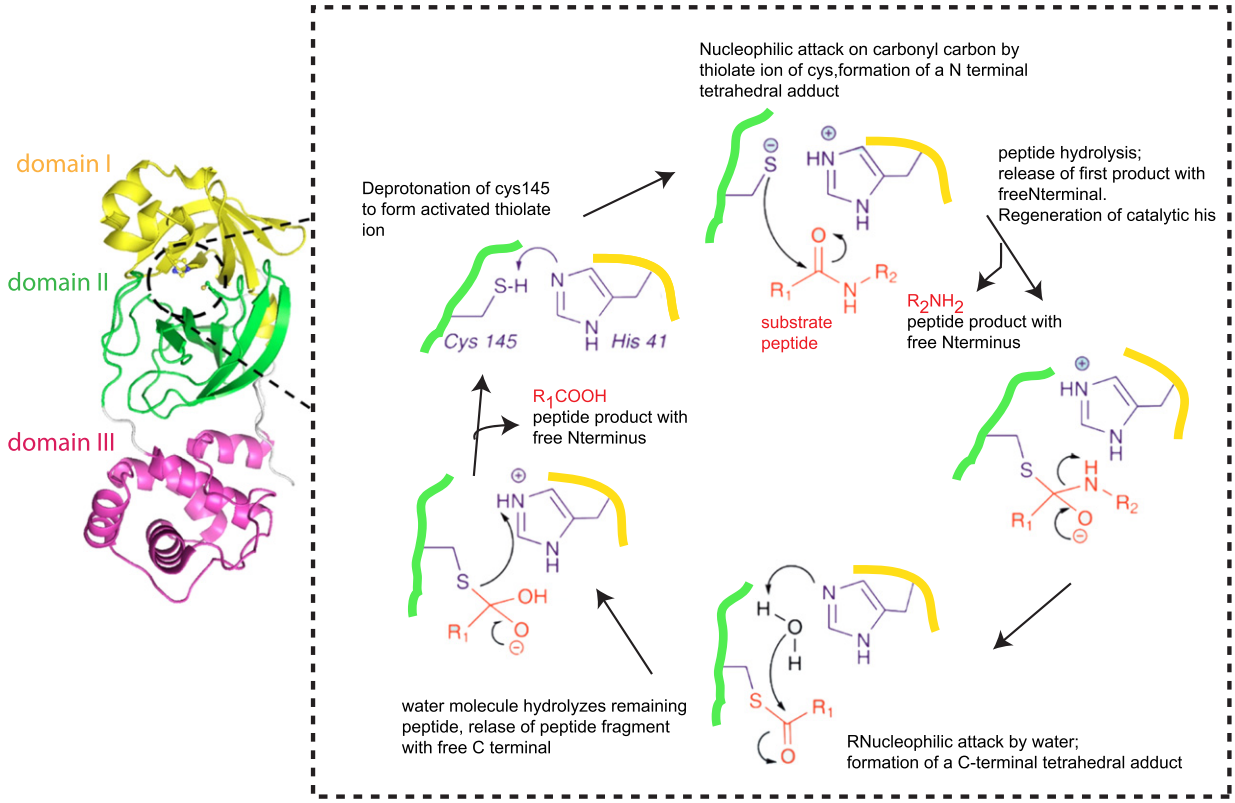

Fig. 2 Structural domains of 3CLpro of SARS-CoV-2 (PDB code 6Y2E) and catalytic mechanism of protease activity.

Structure of 3CLpro

The 3CLpro is a homodimer in its active form. Each monomer has 3 domains: 2 two chymotrypsin-like b-domains (domain I, domain II) comprising anti-parallel beta sheets and a C-terminal α-helical dimerization domain (domain III). The active site is located in the interface of domain I and II and has a catalytic dyad composed of Cys and His residues. The third catalytic element consists of a buried water molecule, which forms H-bonds with the catalytic His residue. During catalysis, His activates the thiol (-SH) group of cysteine, which in turn makes the nucleophilic attack on the carbonyl carbon of the substrate to form a tetrahedral adduct. This is then hydrolyzed by the water molecule present in the catalytic pocket to release the product and regenerate the catalytic His (**Fig. 2**) (Chuck *et al.*, 2013; Jin *et al.*, 2020; Zhang *et al.*, 2020).

Comparison of 3CLpro of SARS-CoV and SARS-CoV-2

The structure of SARS-CoV 3CLpro is 96% homologous to the SARS-CoV-2 enzyme. There is a difference of 12 amino acids between the two proteins. This causes a change in the catalytic site and in the dimerization domain. In SARS-CoV-2, Ser46 is a part of the Cys44-Pro52 loop, which increases the rigidity of the loop, thereby regulating the entry of solvation molecules and inhibitors into the enzymatic cavity. MD simulations showed that the maximal accessible volume (MAV) of the binding cavities is significantly higher in 3CLpro of SARS-CoV compared to SARS-CoV-2. The dimerization domain also has differences in amino acid composition, which alter interactions between protomers. In SARS-CoV, a hydrogen bond between two residues of Thr285, along with hydrophobic interactions between Thr285 and Ile286, are important for dimerization. In SARS-CoV-2 3CLpro, Thr and Ile are replaced by Ala and Leu, respectively (Zhang *et al.*, 2020). This makes the dimer tighter and increases the efficiency of the enzyme.

Effect of 3CLpro on Host Proteins

Recent studies identified several host proteins as substrates of 3CLpro. Viral infections result in upregulation of type I interferons, which leads to transcriptional upregulation of interferon-stimulated genes (ISGs). Several studies have shown that infection with SARS-CoV-2 causes deregulation of the host interferon response. Zhang *et al.* scanned 300 ISGs *in silico* for the presence of 3CLpro target sequences. They validated the top 8 hits and found the E3 ligase RNF20 to be a substrate of the protease in biochemical experiments and in cells overexpressing FLAG tagged RNF20. Further, RNF20 was discovered to be an anti-viral factor, since its depletion increased viral replication. Therefore, apart from activation of nsps, 3CLpro-mediated

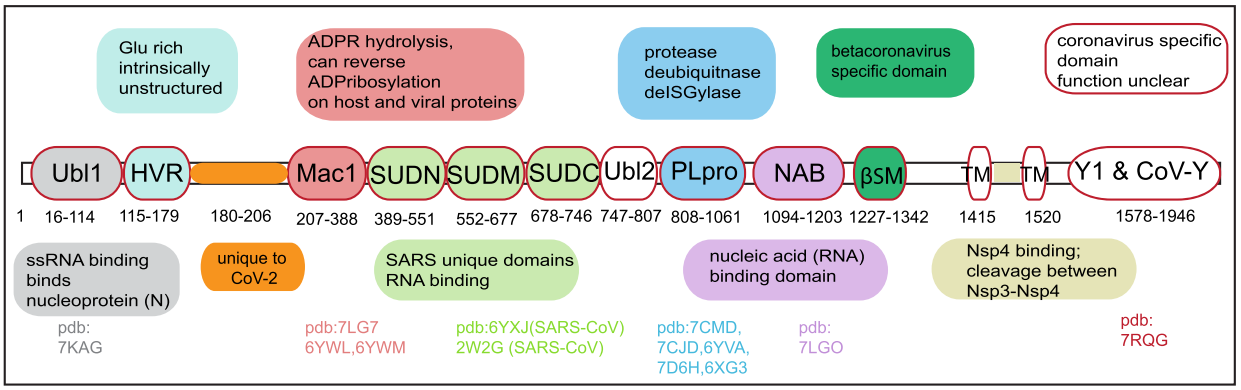

Fig. 3 Domains in NSP3 of SARS-CoV-2 with domain functions and pdb codes of known crystallographic/NMR structures.

cleavage of host proteins may be an important part of its function. Other proteins that can be cleaved by 3CLpro include BIRC6 and SLS25A22 (Zhang *et al.*, 2021).

The Papain-Like Protease (PLpro)

PLpro is encoded by the nsp3 gene. It cleaves the viral pp1a at 3 conserved sites between Nsp1-Nsp4 to generate active Nsp1, Nsp2, and Nsp3. Like the main protease, it is important for the formation of the viral replication/transcription complex (RTC). Apart from activation of viral Nsps, PLpro can also cleave host ubiquitin and ISG15, thereby modulating host metabolism in virus-infected cells. PLpro is found in different coronaviruses, including SARS-CoV, MERS-CoV, TGEV, HCoV-NL63, HCoV 229E and SARS-CoV-2. Some coronaviruses, such as MHV and HCoV-NL63, have 2 PL-proteases, which have different specificities. For example, the PL1pro of MHV cleaves Nsp1↓2 and Nsp2↓3, while the PL2pro cleaves Nsp3↓4 (Kanjanahaluethai and Baker, 2000). HCoV-NL63 PL1pro processes Nsp1↓2 while the PL2pro processes the other two cleavage sites, Nsp2↓3 and Nsp3↓4. SARS-CoV and SARS-CoV-2 both have a single PLpro to process all three cleavage sites, recognizing the consensus cleavage sequence LXGG-XX that is present in Nsp1, Nsp2, Nsp3, as well as the host ubiquitin and ISG15 (Shin *et al.*, 2020). SARS-CoV and SARS-CoV-2 PLpro cleaves peptide bonds between Nsp1 and Nsp2 (LNGG↓AYTR), Nsp2 and Nsp3 (LKGG↓APTK), and Nsp3 and Nsp4 (LKGG↓KIVN) liberating three proteins: Nsp1, Nsp2, and Nsp3.

PLpro is a Part of the Multi-Domain Protein Nsp3

Nsp3 is a multi-domain protein produced by coronaviruses. It consists of 1922 and 1945 amino acids in SARS-CoV and SARS-CoV-2 respectively. Nsp3 is released from pp1a/pp1ab by the papain-like protease, which functions as a domain in Nsp3. It can act as a scaffold to interact with other viral Nsps or host proteins (Imbert *et al.*, 2008; Stukalov *et al.*, 2021). Nsp3 is also important for the formation of RTCs and was identified as one of the major constituents of the double-membrane-spanning pore complex on MHV-induced DMVs (Wolff *et al.*, 2020). Nsp3 contains an N-terminal Ubl (ubiquitin-like) domain, followed by an acidic Glu rich region called the hypervariable region (HVR). This is followed by an a Macrodomain (MAC1, also known as ADP-ribose phosphatase), SUD (SARS-unique domain) domain, and another Ubl domain that is part of PLpro. The C-terminus of the protease has a NAB (nucleic acid binding) domain, followed by a TM (transmembrane segment) and a ZnF (zinc finger) domain. The C-terminus of the protein has Y1 and CoV-Y domains, these are present on the cytosolic side of the ER (**Fig. 3**). Most studies on SARS-CoV and SARS-CoV-2 PLpro have been focused on the isolated PLpro enzyme. However, recent studies show that SARS-CoV-2 PLpro is a more efficient enzyme when it is a part of $Nsp3^{core}$ (amino acids 179–1329 of Nsp3) (Armstrong *et al.*, 2021).

Structure of PLpro

Structural studies on SARS-CoV and SARS-CoV-2 PLpro mainly relied on using the minimal PLpro domain, which was catalytically active (amino acids 1541–1855 of pp1a) (Ratia *et al.*, 2014; Shin *et al.*, 2020). The protein has two domains: an N-terminal ubiquitin-like (Ubl) domain and the catalytic domain. The Ubl domain (amino acids 1–60) consists of five β-strands, one α-helix, and one 310-helix. The catalytic domain has a "thumb–palm–fingers" conformation. The thumb is comprised of six α-helices and a small β-hairpin. The fingers subdomain consists of six β-strands and two α-helices; a zinc ion is coordinated by four cysteine residues located on two β-hairpin loops. The palm subdomain is comprised of six β-strands. The active site with the catalytic residues Cys111, His272 and Asp286 is located at the interface between the thumb and palm subdomains (Osipiuk *et al.*, 2021). The PLpro is a cysteine protease, similarly to the 3CLpro. The active site contains catalytic triad (Cys111, His272, and Asp286), while 3CLpro has catalytic dyad (Cys145 and His41) (Báez-Santos *et al.*, 2014; Kneller *et al.*, 2021). Cys111 acts as a nucleophile, His273 functions as a general acid-base, and Asp287 is paired with histidine to align it and promote deprotonation of Cys111. The thiolate form of Cys111 makes a nucleophilic attack on the carbonyl carbon of the substrate to form a negatively charged

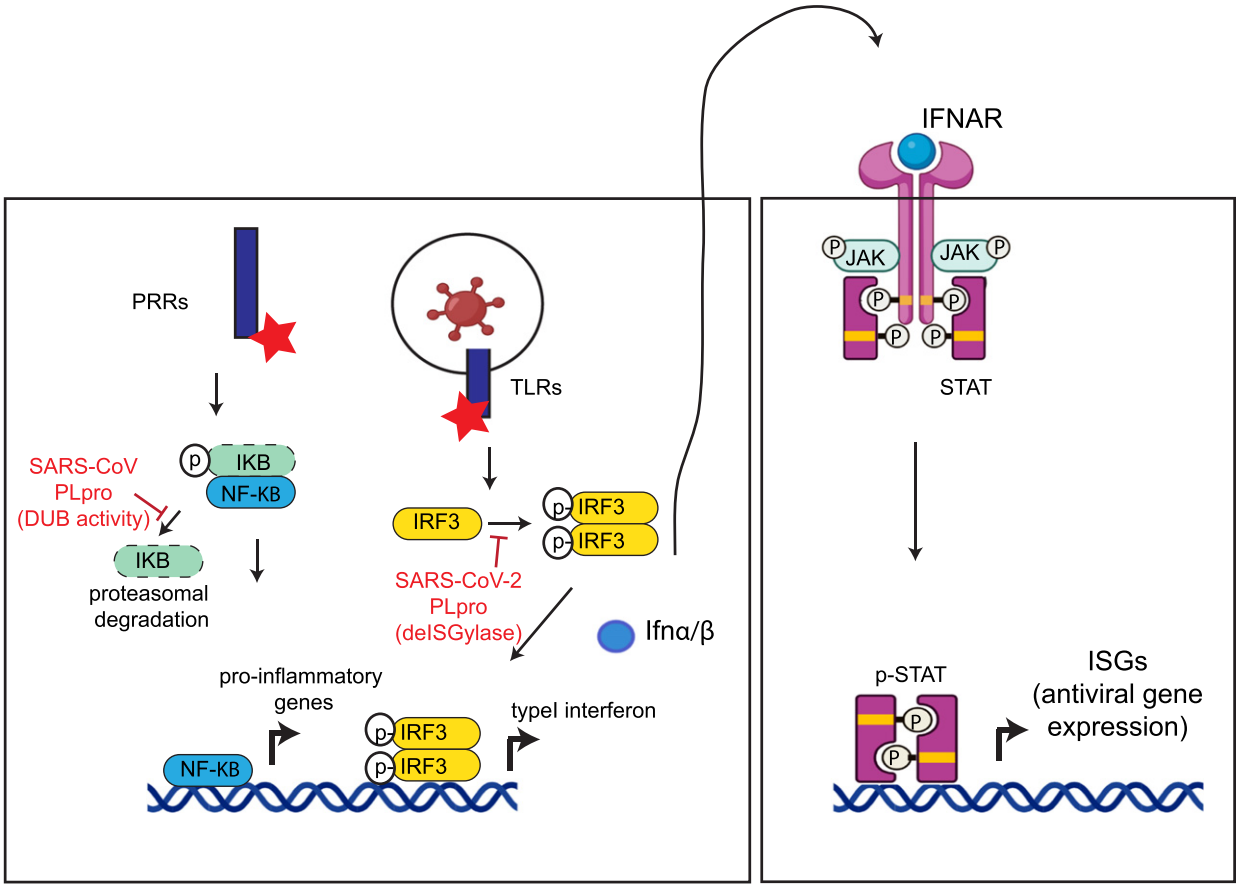

Fig. 4 Innate immune response to viral infection; effect of SARS-CoV and SARS-COV-2 PLpro on NF-KB and IRF3 signaling. Viruses activate pattern recognition receptors (PRRs) which lead to phosphorylation and dimerization of IRF3, causing its nuclear translocation and transcriptional activation of type-I interferon. Type I interferon can activate JAK-STAT1 signaling leading to synthesis of interferon stimulated genes (ISGs).NF-KB pathway is also activated in viral infection which leads to a pro-inflammatory response. SARS-CoV PLpro is a strong deubiquitinase; it inhibits NF-KB signaling by preventing proteasomal degradation of IKB.SARS-CoV-2 PLpro is a strong deISGylase, modulates IRF3 activity by deISGylation.

tetrahedral intermediate. The tetrahedral intermediate is stabilized via the presence of an oxyanion hole adjacent to the active site. The oxyanion of the tetrahedral intermediate is stabilized via Trp107 and Asn110 of the oxyanion hole (Báez-Santos *et al.*, 2015; Ratia *et al.*, 2014). Elimination of the C-terminal amine of the substrate cleaves the peptide bond. A water molecule then hydrolyzes the substrate and the Asp286 of the catalytic triad regenerates the catalytic His residue.

Comparison of PLpro of SARS-CoV and SARS-CoV-2

The SARS-CoV and SARS-CoV-2 enzymes have a similar architecture of active sites and share 83% identity and 90% similarity in sequence. Apart from cleaving the viral pp1a/pp1ab, PLpro is known to cleave host ubiquitin and ISG15. Structural studies on SARS-CoV and SARS-CoV-2 PLpro show differential interaction of the protease with ubiquitin and ISG15. The structure of SARS-CoV PLpro with ubiquitin aldehyde (a semisynthetic ubiquitin with a C-terminal aldehyde group, which can modify the catalytic cysteine and has a 300,000-fold increase in binding affinity relative to unmodified ubiquitin) showed PLpro has a higher preference for binding of K48-Ub2 and ISG15 over K63-Ub2 and mono-ubiquitin (Ratia *et al.*, 2014). From kinetic analyses of SARS-CoV and SARS-CoV-2 PLpros, using activity based probes (consisting of a propargylamide (Prg) "warhead", which forms a covalent bond with catalytic cysteines), it was evident that the SARS-CoV-2 enzyme had higher reactivity with the ISG15–Prg probe, but showed weak activity towards K48-linked di-ubiquitin (K48-Ub_2). On the other hand, SARS-CoV PLpro had the highest reactivity with K48-Ub_2 Prg probes, and less reactivity towards the ISG15–Prg probe (Shin *et al.*, 2020). Experiments with 7-amido-4-methylcoumarine (AMC) or TAMRA tagged Ub and ISG15 probes also showed that the SARS-CoV-2 protease has a stronger preference towards ISG15, while SARS-CoV PLpro preferred K48-Ub2 (Shin *et al.*, 2020; Klemm *et al.*, 2020). The crystallographic structure of SARS-CoV2-PLpro (C111S) and mouse ISG15 (PDB: 6W9C) showed that the N-lobe of ISG15 interacts with the S2 site of the protease. When this structure was compared with the structure of SARS-CoV PLpro in complex with the C-terminus of mouse ISG15 (PDB ID: 5TL7), it was evident that both PLpros share the same binding mode to the C-lobe of the mouse ISG15. On comparing the SARS-CoV2-PLpro–ISG15 complex to that of SARS-CoV-PLpro-K48-Ub2, there was a difference in interactions in

the S2 site of the protease. The S2 binding site in papain-like proteases has variable hydrophobicity at the position that interacts with Ile44 of the distal ubiquitin. SARS-CoV PLpro has Leu76 while SARS-CoV-2 PLpro has Thr75. This may explain the differential interaction of the two PLpros with K48-Ub2 (Shin *et al.*, 2020). The structure of the SARS-CoV-2 PLpro with $ISG15^{CTD}$ (PDB:6XA9) and Ub showed differences in the interactions of the catalytic core with Ub and $ISG15^{CTD}$ that may also help explain the different behavior of the protease towards ubiquitin and ISG15 (Klemm *et al.*, 2020).

Effect of PLpro on Host Processes

Since PLpro can cleave the host cell ubiquitin and ISG15, it may directly affect host metabolism. Many ubiquitin-dependent pathways may be altered in virus-infected cells – especially the pathways which are regulated by K48 linked Ub chains. PLpro is also a deISGylase as it can cleave ISG15 from substrates. ISG15 is produced as a part of the type I interferon response in viral infections. Since both ubiquitin and ISG15 are important in innate immune signaling, the effect of PLpro on NF-κB-dependent gene expression and on type I interferon response has been explored (**Fig. 4**).

Effect of PLpro on NF-κB dependent gene expression

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a family of inducible transcription factors that regulate the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines, and also participates in inflammasome regulation. The canonical NF-κB pathway is activated by diverse stimuli, including ligands binding to cytokine receptors, pattern-recognition receptors (PRRs), TNF receptor (TNFR) superfamily members, T-cell receptor (TCR) and B-cell receptor. This activates phosphorylation and ubiquitination of the NF-κB inhibitor IκBα, causing its proteasomal degradation. This results in rapid and transient nuclear translocation of canonical NF-κB members, predominantly the RelA/p65 and RelB/c-Rel dimers, which can then drive the transcription of genes encoding inflammatory cytokines, chemokines and adhesion molecules. It also regulates expression of genes related to cell proliferation, apoptosis, morphogenesis and differentiation (Oeckinghaus and Ghosh, 2009).

Studies with SARS-CoV PLpro have shown that the deubiquitinating activity of PLpro stabilizes IκBα, and thereby blocks the activation of the NF-κB signaling pathway. This is accompanied by a decrease in the endogenous levels of pro-inflammatory cytokines and chemokines in activated cells (Frieman *et al.*, 2009). In the case of HCoV-NL63 PLpro, these effects of PLpro could be reversed by using the competitive inhibitor GRL-0617 (Clementz *et al.*, 2010). On comparing SARS-CoV and SARS-CoV-2 PLpros, SARS-CoV had a stronger inhibitory effect on the activation of NF-κB. SARS-CoV is a more potent deubiquitinase, which deubiquitinates IκBα, causing its stabilization. Exogenous expression of SCoV-PLpro reduced TNF-induced NF-κB dependent gene expression in a GRL-0617-dependent manner (Shin *et al.*, 2020). Unlike in SARS-CoV-2, the levels of inflammatory cytokines, such as IL6 and IL8, in SARS-CoV-infected cells, is responsive to the PLpro inhibitor GRL-0617, indicating that SARS-CoV PLpro inhibits expression of pro-inflammatory genes. This also highlights how the structural differences in PLproteases translate to functional changes in host signaling.

Effect of PLpro on type I interferon response

The SARS-CoV-2 virus has been shown to inhibit the antiviral interferon response of host cells in several studies (Munnur *et al.*, 2021). The deISGylating activity of PLpro is one of the contributing factors to the inhibition of the type I interferon response of the host.

The immediate response of the host cell to viral infection is to launch the production of type I interferons. Viral nucleic acids (ssRNA, dsRNA, or dsDNA) are recognized by different classes of PRRs, such as the membrane-bound Toll-like receptors (TLRs), the caspase recruitment domain-containing, cytoplasmic RNA helicases, retinoic acid-inducible gene I (RIG-I), or melanoma differentiation-associated gene 5 (MDA5). TLR3 and MDA5 detect viral double-stranded (ds) RNA in the endosomes and cytoplasm, respectively. Intracellular viral RNAs bearing 5′-triphosphates are recognized by RIG-I. These PRRs recruit different adapters leading to the assembly of signaling complexes that relay the signals to the downstream kinase TBK1 (Tank Binding Kinase-1), thus activating IFN regulatory factor-3 (IRF-3). IRF3 is phosphorylated, forms homo-dimers and translocates to the nucleus to regulate transcription of type I interferons (interferon-α, interferon-β). Type I interferons can then amplify the response in an autocrine and paracrine manner by binding to interferon α/β receptors (INFR) which then activate Janus kinase-1 (JAK1), driving the signal transducer and activator of transcription 1 (STAT1)-dependent transcription of anti-viral ISGs. Many viruses, including coronaviruses, have evolved mechanisms to inhibit this antiviral defense mechanism of the host.

SARS-CoV-2 has been shown to deregulate the type-I interferon response. It is often delayed, but hyperactivated in severe Covid-19 patients, characterized by exacerbated inflammation. PLpro-mediated regulation of host cell ISGylation may be one of the many contributing factors leading to this dysregulation. Little is known about the identity of the proteins that are ISGylated in the cell upon viral infection, IRF3 being one of them (Shin *et al.*, 2020). Earlier studies on SARS-CoV showed an inhibition of IRF3 phosphorylation (Devaraj *et al.*, 2007; Matthews *et al.*, 2014). Recent studies show that SARS-CoV-2 PLpro can regulate activation of IRF3-dependent transcription through modulation of it ISGylation. IRF3 ISGylation is evident in cultured Caco2 cells infected with SARS-CoV-2. The proportion of ISGylated IRF3 increases when cells are treated with the PLpro inhibitor GRL-0617. This deISGylating effect of the SARS-CoV-2 protease is stronger than that of the SARS-CoV enzyme. IFNβ dependent transcription is also regulated by PLpro in SARS-CoV-2 infected cells. (Shin *et al.*, 2020).

Development of Inhibitors Against Coronaviral Proteases

Both the main protease and the papain-like protease are good targets for developing drugs. Development of drugs requires access to libraries of uncharacterized compounds, or FDA approved drugs which are already in use and can be repurposed for treatment of coronaviral diseases. Rapid, robust screening platforms are also indispensable for testing compound libraries. Virtual screening using molecular docking can screen much larger compound databases inaccessible to experimental screening. Interaction between the protease and candidate compounds can be tested using computational docking algorithms and molecular dynamics simulations. Activity-based Ub/ISG15 probes and high throughput screening methods using GFP/Luciferase or FRET-based reporters, which are activated in response to cleavage by the protease are important to test the effect of compounds in vitro. Effect of test compounds on viral replication and cytopathic effect are also important parameters to assess in the preclinical stage of drug development.

Molecular Dynamics Simulations and *in silico* Docking Methods

Computational docking is a rapid way of screening huge libraries *in silico* to find compounds that can interact with viral proteases. For this, crystallographic structures of 3CLpro and PLpro are taken from the RCSB protein Data Bank (PDB). Docking of protein ligands onto the rigid protein structure of the protease is usually carried out using software such as DOCK, Rosetta, Glide, AutoDock Vina, and GOLD. Generally, ligands with highest with binding scores are selected for further exploring intra-molecular interactions with the protease. The dynamic behavior of the protease and drug is analyzed using molecular dynamics simulations. Molecular dynamics simulations show the stability of the drug-protease complex over the simulation period in a solvated system. A simulation software package like GROMACS, Desmond, AMBER and CHARMM is used to put the complex in a physiological-like system. The dynamics of the system is computed by numerically solving Newton's equations of motion using force field parameters. Later, interaction energies can be calculated over the simulation period to analyze the stability of the drug in the binding site of the proteases (Huynh *et al.*, 2021).

Kinetic Analysis of Protease Activity in Presence of Inhibitors

Activity-based Ub/ISG15 probes with a propargyl warhead that covalently attaches to the active site cysteine, can be incubated with the protease in presence of different concentrations of candidate compounds. The attachment of probes to the protease can be analyzed on a silver-stained polyacrylamide gel. However, this read-out is unsuitable for screening large libraries of compounds. Use of Ub/ISG15-AMC is a better read-out for high-throughput screens. Ub-AMC or ISG15-AMC is cleaved by the protease to cause an increase in AMC fluorescence which can be measured in a fluorescent plate reader and is a measure of protease activity. Fluorescence can be measured over time to calculate kinetic parameters of the enzyme (Shin *et al.*, 2020; Klemm *et al.*, 2020).

FlipGFP Based Protease Activity Assays

Froggatt *et al.* (2020) developed a FlipGFP based assay to detect 3CLpro activity. In this assay, the 10th and 11th β-strands of the green fluorescent protein (GFP) are expressed separately from, and in a conformation incompatible with, the rest of the GFP beta-barrel. A linker containing a cleavage site of 3CLpro holds the two GFP beta-strands, β10–11, in an inactive, parallel conformation. In the presence of active protease, the linker containing the cleavage site is cut. This cleavage allows GFP β11 to re-orient such that GFP β10–11 are antiparallel and able to fit into GFP β1–9, inducing an increase in GFP fluorescence. SARS-CoV-2 3CLpro activity and the effect of the 3CLpro inhibitor GC376 was measured with this assay in this study (Froggatt *et al.*, 2020).

Luciferase Based Reporter Systems

Luciferase-based reporters to test protease activity include constructs expressing a firefly luciferase maintained in an inactive form by a consensus cleavage site for the viral proteases so that the luminescent biosensor is turned on upon $3CL^{Pro}$ expression or SARS-CoV-2 infection. Treatment with a drug/inhibitor of viral proteases decreases the luciferase signal. Cell lines stably expressing these constructs can be used for high-throughput screening of libraries of FDA approved compounds, protease inhibitors and biologically active compounds.

FRET-Based Assays

FRET (Fluorescence resonance energy transfer)-based probes are designed by inserting the cleavage sequence of the protease between two fluorophores, which are FRET pairs (for example CFP and YFP). In the absence of the protease, excitation of the first fluorophore leads to energy transfer to the second fluorophore, which then emits fluorescence. Cleavage by the protease separates the FRET pair, reducing the fluorescence emission of the second fluorophore. Therefore, protease activity is measured by a decrease

in the fluorescent signal and effective inhibitors prevent this decrease (Chuck *et al.*, 2010). The second type of such reporters include dark-FRET reporters that have a fluorescent donor group linked to a quencher dye by means of the protease cleavage site. In the absence of the protease, the substrate fluorescence is quenched by the two dyes being in close proximity of one another. In the presence of the protease, the internally quenched substrate is hydrolyzed, producing a highly fluorescent peptide and the fluorescence increase is proportional to the coronavirus protease activity. The presence of inhibitors decreases the fluorescence of these reporters (Ma *et al.*, 2020).

Pharmacokinetics Analysis

A good drug must meet the conditions of absorption, distribution, metabolism, excretion and toxicity (ADMET). For compounds that have been assessed in terms of pharmacokinetics before, ADME assessment of selected molecules were performed through the freely available online SwissADME web tool, and toxicity predictions were assessed using the pkCSM server (Daina *et al.*, 2017).

Viral Replication and Cytopathic Effect

Viral replication can be assessed by infecting cultured cells with the virus in the presence or absence of the candidate compounds. Cultured cell lines usually used for SARS-CoV and SARS-CoV-2 infection include Vero E6, Caco-2 and A549. Viral replication is assessed by a plaque formation assay. Here, different dilutions of the virus are allowed to infect cultured cells growing in a mono-layer. After adsorption of the virus onto the cell surface, an immobilizing overlay is used to cover the infected monolayer, to prevent virus spread and restrict virus growth to foci of cells at the sites of initial infection. Killing of cells leads to clearings in the monolayers, which form plaques. The number of plaques can be counted as a measure of virus induced death. Viral replication can also be measured by Real Time-PCR from infected cells, using primers against viral subgenomic RNAs. The effect of test compounds on viral infection is also tested by measuring the cytopathic effect (CPE) in the presence of different concentrations of the compound (Shin *et al.*, 2020).

Inhibitors of 3CLpro

The effort to develop 3CLpro inhibitors began after the SARS epidemic in 2002–2004. Comparing amino acid sequence and structures of the 3CLpros of SARS-CoV-2, SARS-CoV and MERS-CoV shows a high degree of sequence and structural similarity. These made it possible to test inhibitors developed against 3CLpro of SARS-CoV against that of SARS-CoV-2. These inhibitors can be classified as peptidomimetic inhibitors and small-molecule inhibitors.

Peptidomimetic Inhibitors

Peptidomimetic inhibitors consist of peptides, which mimic natural peptide substrates of 3CLpro and a warhead group, which is spatially very close to the catalytic cysteine of the active site. The peptide forms non-covalent interactions with the protease, like the natural substrate, while the warhead undergoes a nucleophilic attack to form an irreversible covalent bond with the cysteine. These warheads contain different functional groups like aldehydes, ketones, benzothiazolones, and ketoamides. The initial 3CLpro inhibitors were developed by Pfizer, Cytovia, and Agouron (Chia *et al.*, 2021). These had 1–3-residue peptidomimetics, where the P1 glutamine was modified to either 5- or 6-membered lactams and had a C-terminal electrophilic α,β-unsaturated ester warhead. Some of these compounds were originally designed to inhibit the rhinovirus 3 C protease, which shares some structural similarity to coronavirus 3CLpro. The compound PF-00835231 was developed by Pfizer as an inhibitor for SARS-CoV 3CLpro following the SARS epidemic in 2002 (Boras *et al.*, 2021). This was also found to be effective against SARS-CoV-2 3CLpro since the enzymes are 96% identical. It is administered intravenously as a phosphate prodrug (called PF-07304814) and is in clinical trials.

The 3CLpro inhibitor PF-07321332 (Nirmatrelvir) was successfully designed and tested as an oral drug for Covid-19 by Pfizer. It is co-administered with a low dose of ritonavir to increase its half-life and maintain appropriate systemic concentration of the drug. This Nirmatrelvir + Ritonavir combination was named PAXLOVID, and was in phase 2/3 clinical trials [called EPIC-HR (Evaluation of Protease Inhibition for COVID-19 in High-Risk Patients)] which was conducted on non-hospitalized subjects above the age of 18 with confirmed COVID-19, and who are at an increased risk to develop the severe illness. The data demonstrated an 89% reduction in the risk of COVID-19-related hospitalization or death in adults treated with PAXLOVID, compared to those receiving a placebo, within three days of symptom onset (primary endpoint). The U.S. Food and Drug Administration (FDA) recently authorized the emergency use of PAXLOVID™ for the treatment of mild-to-moderate COVID-19.

Small Molecule Inhibitors

Non-peptidic small-molecule inhibitors usually do not bind covalently in the active site, but instead interact with residues in the S1′, S1, S2 and S4 pockets via non-covalent interactions (hydrogen bonds, hydrophobic bonds and van der Waals forces), and competitively inhibit the substrate from binding. GC376 is a pre-clinical dipeptide-based protease inhibitor that was previously used for treating feline infectious peritonitis virus (FIPV) (Froggatt *et al.*, 2020). It is a 3CLpro inhibitor which is in pre-clinical

trials. GC376 was further modified with a piperidine moiety to improve the IC50 of the drug (Kankanamalage *et al.*, 2018). A high-throughput screening of National Institute of Health (NIH) compound libraries (approximately 293,000 compounds) was performed to identify 3CLpro inhibitors. From here, pyridyl derivatives of dipeptides were subsequently optimized as strong inhibitors of SARS-3CLpro (Liu *et al.*, 2020a,b).

Natural Compounds and Repurposed Drugs

In addition to small molecules, natural products such as Quercetin, epigallocatechin gallate, and gallocatechin gallate (GCG) also inhibit SARS-CoV 3CLpro, with IC50 in the range of 40–80 μM (Nguyen *et al.*, 2012). The HCV NS3–4A inhibitor Boceprevir, and calpain inhibitors II, and XII have been repurposed as 3CLpro inhibitors (Ma *et al.*, 2020). Bicycloproline derivatives of boceprevir or telaprevir, can inhibit SARS-CoV-2 Mpro activity in vitro, with IC50in the range of 6 nM and 748 nM and have been reported to exhibit antiviral activity in mice (Qiao *et al.*, 2021). HIV protease inhibitors Lopinavir, Ritonavir, Darunavir have also been found to be effective in inhibiting SARS-CoV 3CLpro. They are now in clinical trials.

Inhibitors of PLpro

Initial efforts to develop inhibitors against SARS-CoV PLpro identified 2 thiopurine compounds: 6MP and 6- TG that were used as drugs for leukemia. High toxicity prevented their further use as drugs for SARS. Although PLpro is a cysteine protease, much like 3CLpro, it is difficult to target it with covalent inhibitors that modify the active site cysteine because it has a featureless P1 and P2 sites with 2 glycine residues. Peptide-based covalent inhibitors of PLpro (VIR250 and VIR251) showed weak potency with IC50 values of 50 μM in biochemical assays (Rut *et al.*, 2020).

The first successful inhibitor was GRL-0617, which was discovered in a screen with 10,000 compounds. It has an IC50 in the range of 0.6–0.8 μM. The ability of PLpro to cleave Ub-AMC was used as a readout of PLpro activity (Ratia *et al.*, 2008). GRL-0617 is a non-covalent inhibitor and can restrict viral replication in Vero E6 cells infected with SARS-CoV and SARS-CoV-2 (Ratia *et al.*, 2008; Shin *et al.*, 2020). GRL-0617 could rescue the deISGylation of IRF3 in SARS-CoV-2-infected Caco2 cells (Shin *et al.*, 2020). GRL-0617 lacked the potency for clinical use, but the structure of SARS-CoV–GRL-0617 complex helped refine the design of PLpro inhibitors. Rac5c, rac3j, rac3k are 3 compounds that bind in the channel occupied by the ubiquitin/ISG15C-terminal tail, present between the SARS-CoV PLpro Thumb domain and a so-called Blocking Loop (BL). The BL sequence, and length, are conserved between SARS and SARS-Cov-2 PLpro and can inhibit PLpro of both viruses (Báez-Santos *et al.*, 2014; Klemm *et al.*, 2020). Shen *et al*. developed 2-phenylthiophene compounds (XR8–23, XR8–24) with nanomolar inhibitory potency. Cocrystal structures showed that these compounds bind at a site that is distal to the active site cysteine, inducing a conformation change in a β hairpin loop (BL2 loop), which blocks the substrate from entering the active site. These compounds have slow off-rates, and low micromolar antiviral potency in SARS-CoV-2-infected human cells (Shen *et al.*, 2021).

New Drugs to Target Viral Proteases: Nanobodies, Reversible Covalent Inhibitors, and PROTACs

A recent study showed that PLpro activity can be inhibited by competitive nanobodies that bind to PLpro at the substrate binding site with nanomolar affinity thus inhibiting the enzyme (Armstrong *et al.*, 2021). These inhibitors are noncovalent or covalent. Non-covalent inhibitors show weak binding to the active site, reducing the risk of non-specific interaction and have lower toxicity than covalent inhibitors. Covalent inhibitors show stronger irreversible binding to the active site but are more toxic, and unsuitable for long-term use. New drugs may be developed as reversible covalent inhibitors that have advantages of both covalent and non-covalent inhibitors. To make a reversible covalent inhibitor targeting a viral protease, the warhead of an existing inhibitor will be replaced with a cyano group, which will form a reversible covalent bond with the active site cysteine residue. A reversible covalent bond will make more stable contacts with the enzyme than a non-covalent inhibitor, but will be less permanent than an irreversible inhibitor. Proteolysis targeting chimeras (PROTACS) are also being developed to inhibit viral proteins. PROTAC technology can be used in the design of anti-coronavirus drugs, which can cause targeted degradation of viral proteins in virus infected cells. The principle of PROTAC technology involves the use of a bifunctional small molecule that links the target protein and the E3 ligase, facilitating its ubiquitination and proteasomal degradation. To date, the PROTAC DGY-08–097 has been used successfully to degrade the HCV protein NS3/4A efficiently. To develop a PROTAC against a viral protease, a reversible covalent inhibitor has to be coupled to a PROTAC molecule. This would allow the inhibitor to come in contact with the active site cysteine, to form a reversible covalent bond. This close proximity would initiate ubiquitination by the E3 ligase component of the PROTAC followed by degradation of the viral protease.

Conclusion

The COVID-19 pandemic has been a huge impetus for a change in biological research. Research has been fast-paced, collaborative, data and reagents have been shared openly, findings are being evaluated, assessed and criticized in real time. Research

on coronaviruses has been at the forefront of this enterprising attitude. This review summarizes available literature on the coronaviral proteases 3CLpro and PLpro. Most of the research that has been reviewed here represents new discoveries that have been published in the last two years. Our current understanding of the structure and function of coronaviral proteases makes it possible to design and screen inhibitors, and utilize new technology with the final goal of translating laboratory findings to the clinic.

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