Innate immune evasion mediated by picornaviral 3C protease: Possible lessons for coronaviral 3C-like protease?

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Funding information
Heart and Stroke Foundation of Canada, Grant/Award Numbers: G-16-00013800, G-18-0022051; Canadian Institutes of Health Research, Grant/Award Numbers: PJT 159546, PJT-173318; Natural Sciences and Engineering Research Council of Canada, Grant/Award Number: RGPIN2016-03811

Summary
Severe acute respiratory syndrome coronavirus-2 is the etiological agent of the ongoing pandemic of coronavirus disease-2019, a multi-organ disease that has triggered an unprecedented global health and economic crisis. The virally encoded 3C-like protease (3CLpro), which is named after picornaviral 3C protease (3Cpr), due to their similarities in substrate recognition and enzymatic activity, is essential for viral replication and has been considered as the primary drug target. However, information regarding the cellular substrates of 3CLpro and its interaction with the host remains scarce, though recent work has begun to shape our understanding more clearly. Here we summarized and compared the mechanisms by which picornaviruses and coronaviruses have evolved to evade innate immune surveillance, with a focus on the established role of 3CLpro in this process. Through this comparison, we hope to highlight the potential action and mechanisms that are conserved and shared between 3CLpro and 3CLpr. In this review, we also briefly discussed current advances in the development of broad-spectrum antivirals targeting both 3CLpro and 3CLpr.

KEYWORDS
Covid-19, picornaviruses, SARS-CoV-2, 3CLpro, 3CLpr

Received: 15 October 2020 | Revised: 3 December 2020 | Accepted: 4 December 2020
DOI: 10.1002/rmv.2206

Abbreviations: ACLY, ATP-citrate synthase; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; avoSs, antiviral stress granules; CARD, caspase activation and recruitment domain; cGAS, cyclic GMP-AMP synthase; Covid-19, coronavirus disease-2019; CVaA6/A6/B3, Coxackievirus-A6/A6/B3; CYLD, cylindromatosis; CALCOCCO2, coiled-coil domain-containing protein 2; DAMPs, danger associated molecular patterns; DCP1/2, decapping protein ½; DDX6, DEAD box helicase 36; DHX5, death domain-helix box 36; dsRNA, double-stranded RNA; eIF4E/F/G, eukaryotic initiation factor 4E/F/G; EDC3/4, enhancer of mRNA decapping protein 3/4; EMCV, encephalomyocarditis virus; ERAV, equine rhinitis A virus; ERV7/A71/D68, enterovirus-A71/D68; FMDV, foot-and-mouth disease virus; GBP1, oligo-specific brevetoxin A resistance guanine nucleotide exchange factor; G3BP1, Ras-GTPase-activating SH3 domain binding protein 1; GSDMD, gasdermin D; HAV, hepatitis A virus; HDAC2, histone deacetylase 2; HRV, human rhinovirus; hnRNP M/K, heterogeneous nuclear ribonucleoprotein M/K; IFN-κB, type 1 interferon; IKK, IkB kinase-e; IRES, internal ribosomal entry site; IF3/5/7, interferon regulatory factor-3/5/7; IGSs, interferon-stimulating genes; K63-linked, lysine-63-linked; M protein, membrane/matrix protein; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein-5; MERS-CoV, Middle East respiratory syndrome-CoV; MEX3C, Mex-3 RNA binding family member C; mRNPs, messenger ribonucleoproteins; NDP52, nuclear dot 10 protein 52; NLRP3, NLR family pyrin domain containing protein 3; N proteins, nucleocapsid proteins; NEMO, NF-κB essential modulator; NFκB, nuclear factor-κB; NLRs, (NOD)-like receptors; NS5P, nonstructural protein 5; ORF1a/1b, open reading frame 1 a/b; PAMPs, pathogen-associated molecular patterns; PDCoV, porcine deltacoronavirus; PDCoD6IP, programmed cell death 6-interacting protein; PEDV, porcine epidemic diarrhea virus; PFAS, phosphoribosylformylglycinamidine synthetase; PKR, protein kinase R; PLEKH1M, pleckstrin homology domain and RUN domain containing M1; PRRs, pathogen recognition receptors; PRRSV, porcine reproductive and respiratory syndrome virus; PLpro, papain-like protease; PV, poliovirus; RIP1, receptor-interacting serine/threonine-protein kinase 1; RLRs, RIG-I-like receptors; RIGp, RNA-dependent RNA polymerase; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; SNAP29, synaptosomal-associated protein-29; sRNA, single-stranded RNA; STAT2, signal transducer and activator of transcription 2; STX17, syntaxin 17, SQSTM1, sequestosome 1; SVV, Seneca Valley virus; TAB1, TAK1 binding protein-1; TAIL5, terminal amine isonicotinic labeling of substrates; TBK1, TANK binding kinase-1; TLRs, toll-like receptors; TRAF3, TNF receptor associated factor-3; TRIM25, tripartite motif containing 25; TRIF, (TIR)-domain-containing adapter-inducing interferon-β; TRMT1, tRNA methyltransferase 1; UMP, uridine monophosphate; UTR, untranslated region; VAMP8, vesicle-associated membrane protein 8; VPg, viral genome-linked; XRN1, 5′-3′ exoribonuclease 1; 2Aττττ, 2A-pro tease; 3CLpro/3Cpr; 3C-like protease/3C protease; 3Dpoly, 3D polymerase.
1 INTRODUCTION

Coronavirus disease-2019 (Covid-19) is a multiple organ disease that has posed an unprecedented health and economic threat worldwide since its emergence in late 2019. Covid-19 is caused by a novel virus strain, \(^2\)namely severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) \(^3\), categorized within the family Coronaviridae. It can infect various hosts and target multiple organs through the body. \(^4\)Coronaviruses are a family of enveloped, single-stranded RNA (ssRNA) viruses with positive polarity that can cause respiratory, enteric, cardiovascular, and central nervous system diseases. \(^5,6\) This family of RNA viruses features the second largest genome size (27–31 kb) found to date right after planarian nodovirus (~41.1 kb). \(^7\) Together with SARS-CoV and Middle East Respiratory Syndrome-CoV (MERS-CoV) that caused SARS and MERS outbreaks in 2003 and 2012, respectively, SARS-CoV-2 belongs to the genus betacoronavirus. The genome of betacoronavirus encodes more than 20 proteins, including four major structural proteins (i.e., a spike (S) protein that binds to the cell receptor and mediates fusion between virus and cell membrane, a small envelope (E) protein, a highly hydrophobic membrane (M) protein, and a nucleocapsid (N) protein that interacts with viral RNA to form a helical nucleocapsid structure), two cysteine proteases (i.e., a papain-like cysteine protease (PLpro) and a 3-chymotrypsin-like cysteine protease (3CLpro), also known as the main protease, M\(_{\text{pro}}\) that processes viral polyproteins into individual functional proteins, a helicase required for unwinding double-stranded RNA (dsRNA), a RNA-dependent RNA polymerase (RdRp) that catalyzes the replication of RNA from RNA template, and other enzymes such as endo- and exonucleases essential for viral nucleic acid metabolism. \(^8\) Among these proteins, SARS-CoV-2 proteases play a vital role in viral replication and transcription, thereby being recognized as attractive antiviral targets for Covid-19 treatment. \(^9,10\) Of the two known CoV proteases that are encoded by open reading frame 1a (ORF1a), 3CLpro [corresponding to nonstructural protein 5 (NSP5)], which is highly conserved among all CoV 3CLpro, has been identified to be structurally analogous to the 3\(_\text{Cpro}\) of picornaviruses (3\(_\text{Cpro}\)) is named after the picornaviral 3\(_\text{Cpro}\)). \(^11,12\) Despite subtle structural differences in the active sites, 3CLpro and 3\(_\text{Cpro}\) share a similar chymotrypsin-like tertiary structure with a catalytic triad (or dyad) site containing a cysteine nucleophile (Figure 1). Moreover, both of the enzymes have a strong preference for glutamine (Gln) at the P1 position of their targets, the most key determining factor for their substrate recognition. The conserved active sites of 3\(_\text{Cpro}\) and 3CLpro have been confirmed by high-resolution three-dimensional structural analysis. Therefore, it is proposed to serve as an attractive target for the design of broad-spectrum antiviral drugs. \(^13–15\) Picornaviruses are small, non-enveloped viruses containing a positive-sense, ssRNA genome with a length of 7.0–8.5 kb. This family comprises 29 genera, including Aphthovirus (e.g., foot-and-mouth disease virus, FMDV), Cardiovirus (e.g., encephalomyocarditis virus, EMCV), Enterovirus (e.g., poliovirus, PV; coxsackievirus A16/B3, CVA16; CVB3; enteroviruses A71/D68, EV-A71; EV-D68), Rhinovirus (e.g., human rhinovirus, HRV), and Hepatovirus (e.g., hepatitis A virus, HAV) genera. \(^16\) Picornavirus genomic RNA at its 5′ end is covalently linked to a small viral protein (VPg, also known as 3B) that serves as a primer for the initiation of viral RNA replication. Further, instead of a cap structure, the genome of picornaviruses possesses an element termed internal ribosome entry site (IRES) in their 5′-untranslated region (UTR), which is necessary for initiating a cap-independent translation of viral RNA. The viral genome of picornaviruses contains one open reading frame encoding a single viral polyprotein that undergoes proteolysis by two viral proteases, 2A\(_\text{pro}\) and 3\(_\text{Cpro}\), with the latter being responsible for the majority of the maturation cleavage events of viral polyprotein similar to coronaviral 3CLpro. \(^17\) In addition to processing viral polyprotein, picornaviral proteases also target cellular proteins to evade the human immune surveillance and facilitate viral infection. \(^18\)

Given the common characteristics of 3\(_\text{Cpro}\) and 3CLpro, we postulate that SARS-CoV-2, like picornaviruses, is capable of regulating host innate antiviral processes through the catalytic activity of its 3\(_\text{Cpro}\). The delay or inhibition of multiple host antiviral machineries would allow effective viral growth and subsequently optimal release and infection. Here we will recapitulate some of the scenarios on how picornaviruses utilize its 3\(_\text{Cpro}\) to target major host antiviral mechanisms.

1.1 Structural and functional similarities between picornaviral 3\(_\text{Cpro}\) and coronaviral 3CLpro

Early during the viral replication cycle, the positive-sense ssRNA (+ssRNA) genomes of picornaviruses and coronaviruses are translated into one or more polyproteins, which include integrated viral protease domains. Maturation cleavage events mediated by virally encoded proteases in both backgrounds are indispensable for virus replication. The picornaviral 3\(_\text{Cpro}\) (working in concert with the 2A\(_\text{pro}\)) mediates the majority of viral cleavage events including autocleavage from the 3D polymerase (3D\(_\text{pro}\)) domain of the virus. Similarly, coronaviral 3CLpro is responsible for at least 11 maturation cleavages of the viral replicase polyproteins, including its own autoproteolytic cleavage. Beyond these requisite viral polyprotein cleavages, both 3\(_\text{Cpro}\) and 3CLpro target and cleave host cellular proteins. Due to the importance in their respective viral backgrounds, these proteases have been extensively studied as primary targets for viral inhibition for over 30 years.

The picornaviral 3\(_\text{Cpro}\) is a chymotrypsin-like cysteine protease comprised of two β-barrel domains of six antiparallel strands which surrounds a conserved Cys-His-Asp/Glu catalytic triad (Figure 1). \(^19,20\) The protease preferentially cleaves after a P1-Gln with greater cleavage site variability in the other cleavage site residue positions. \(^21\) During picornaviral replication, the 3D\(_\text{pro}\) precursor, which is able to process the P1 structural precursor but lacks polymerase activity, is cleaved to release 3\(_\text{Cpro}\) and viral RdRp 3D\(_\text{pro}\). The conversion of 3D\(_\text{pro}\) to 3\(_\text{Cpro}\) plays a critical role in facilitating the transition and regulation from viral translation to replication. \(^22\) Structurally and functionally analogous to the picornaviral 3\(_\text{Cpro}\), the coronaviral
3CL\textsuperscript{pro} is an approximately 300 residue, 3-domain protease. Domains 1 and 2 comprise the substrate-binding and enzymatic active sites of the protease with dimerization driven by interactions between the structurally unique and largely helical domain.\textsuperscript{25,26} Domains 1 and 2 of 3CL\textsuperscript{pro} form a chymotrypsin-like fold comprised of antiparallel \( \beta \)-barrels housing the His-Cys catalytic dyad residues.\textsuperscript{26,27} The antiparallel \( \beta \)-barrel conformation within domains 1 and 2 surrounding and forming the active site of 3CL\textsuperscript{pro} shares structural similarity to the core structure of the picornaviral 3C\textsuperscript{pro}, albeit with subtle differences in strand positioning (Figure 1). Unlike the picornaviral 3C\textsuperscript{pro} (monomer with only two catalytic domains), an attached helical third-domain in 3CL\textsuperscript{pro} facilitates dimerization of the protease, an essential event for its enzymatic activity and viral replication (Figure 1C, Domain 3).\textsuperscript{28} While targeting the third domain of 3CL\textsuperscript{pro} serves as a valid strategy to disrupt its dimerization and catalytic activity, any inhibitors identified or designed to do so likely have no impact on picornaviral 3C\textsuperscript{pro} activity since dimerization is not necessary for its function. In addition, among coronaviruses, there is considerably more structural conservation for the chymotrypsin-like fold of 3CL\textsuperscript{pro} compared to that of the picornaviral 3C\textsuperscript{pro}. Based on large part to their structural and functional similarities between the 3C\textsuperscript{pro} and 3CL\textsuperscript{pro}, it remains unclear to what extent these proteases share common host cellular targets during viral replication and what role these potentially shared cleavages play in viral replication.

2 | THE SUBVERSION OF HOST DEFENSE MECHANISMS BY PICORNAVIRAL 3C\textsuperscript{PRO}

2.1 | Targeting type I interferon signaling pathway

The effectiveness of an antiviral innate immunity depends on the accurate recognition of viral moieties, known as the pathogen-associated molecular patterns (PAMPs), by pattern-recognition receptors (PRRs) composed of at least three classes: retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).\textsuperscript{29} Upon RNA viral infection, dsRNAs often accumulate in cells in the form of the viral genome or its replication intermediates. The dsRNAs can be recognized by cytosolic viral RNA sensor, RLRs (e.g., RIG-I (encoded by Ddx58 gene) and melanoma differentiation-associated proteins (MDA5, encoded by Ifih1 gene)), and/or endosomal
viral RNA sensor (e.g., TLR3) to initiate type I interferon (IFN-I) immune response. While RIG-I preferentially binds to shorter dsRNA (<1-2 kb) bearing 5’-triphosphate group, MDA5 primarily recognizes long dsRNA. Similar to picornavirus, SARS-CoV-2 has a long RNA genome. It is therefore expected that SARS-CoV-2 RNA favorably binds to MDA5 rather than its paralog RIG-I. Indeed, MDA5 has been previously shown to be the specific PRR that recognizes murine coronavirus RNA. Interaction between viral RNA and MDA5 forms MDA5 filament along dsRNAs, which brings together neighboring caspase activation and recruitment domain (CARD) in close proximity to induce oligomerization and activation of the adapter mitochondrial-antiviral signaling protein (MAVS). Activated MAVS then transmits the signals to its downstream transcription factors, interferon regulatory factor-3/7 (IRF3/7) and nuclear-factor-κB (NF-κB), through TANK-binding kinase-1 (TBK1) and IκB kinase-ε (IKKe). The homo- or hetero-dimerized IRF3/7 subsequently translocate to the nucleus and induce the expression of IFN-I-associated genes (Ifna and Ilnb1), which could further activate the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling cascade to trigger the expression of antiviral genes, called interferon-stimulated genes (ISGs). Please see Figure 2 for the details.

To antagonize human antiviral innate immune response, picornaviruses target critical components of the RLR signaling pathway for degradation. It was reported that infections with Seneca Valley virus (SVV), PV, EV-A71, EV-D68, CBV3, CVA16, CVA6, HRV-1A, EMCV, and HAV cleave MDA5, MAVS, RIG-I, IRF7, and/or IRF9 through the actions of 3Cpro, leading to a disruption of RLR-mediated IFN-I immune responses. In addition to 3Cpro, studies have found that MDA5 and MAVS can also be targeted by 2Apro upon PV, EV-A71, CBV3, and HRV-1A infections. Moreover, both TAK1 binding protein-1 (TAB1) and NF-κB essential modulator (NEMO), an adapter protein bridging the canonical IKKα/β kinases and the noncanonical kinases TBK1/IKKe via the TANK adapter (TA), are cleaved by 3Cpro following EV-A71, FMDV and HAV infections, resulting in reduced production of IFN-I. In addition, FMDV utilizes its 3Cpro to inhibit STAT2 function, a component of the IFN-stimulated gene factor 3 complex, which is also mirrored by the 3CLpro of PDCoV (Table 1, yellow highlighted row). Another interesting finding associated with this topic is 3Cpro-induced cleavage of Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) during CBV3, EV-A71, and HAV infections. TRIF is an adapter protein mediating type I IFN antiviral response downstream of the endosomal TRIF3 (a viral RNA sensor) and cleavage causes the loss of its function in host defense.

In addition to directly targeting components of the IFN-I pathway, 3Cpro can also modulate the function of proteins that participate in the regulation of this pathway. For instance, the 3Cpro of EV-A71 downregulates miRNA-526a, consequently leading to increased expression of cylindromatosis (CYLD, a target of miRNA-526a). CYLD is a deubiquitinating enzyme negatively regulating the function of RIG-I by removing K63-linked polyubiquitin chains from RIG-I. Ubiquitination is a post-translational modification required for RIG-I activation, and deubiquitination results in its inactivation. Together, available evidence reveals that 3Cpro plays a key role in the effects of picornaviruses in counteracting the host antiviral immune response by cleaving or inactivating essential adapter proteins in the RIG-I/MDA5-MAVS and/or the TLR3-TRIF signaling pathways (Figure 2).

Like picornaviruses, both SARS-CoV and MERS-CoV trigger a limited IFN-I response. A mouse model infected with SARS-CoV demonstrated that a significant delay in IFN production contributes to disease progression and severity. Using different model systems including cells and ferrets infected with SARS-CoV-2 and post-mortem lung tissues from COVID patients, a recent study showed that SARS-CoV-2 infections elicit low levels of IFN-I and no activation of TBK1 and ISGs. Of note, it was found that SARS-CoV-2 is highly sensitive to IFN-I, suggesting an important role for IFN-I in antiviral defense. Although experimental data on SARS-CoV-2 is still limited, current evidence from SARS-CoV and MERS-CoV research revealed that CoVs develop different strategies to overcome the host innate immunity. For example, the PLpro of SARS-CoV, which has deubiquitinating activities, acts as an IFN-I antagonist by removing ubiquitin chains from IRF3 and through preventing the phosphorylation of IRF3. It was also discovered that the ORF3b, ORF6, and N proteins of SARS-CoV inhibit production and action of IFN-I. Moreover, M protein of SARS-CoV was shown to physically associate with RIG-I, TBK1, IKKe, and TRAF3 and inhibit gene transcription of IFN-I. Most interestingly, several coronaviruses, including SARS-CoV-2, porcine deltacoronavirus (PDCoV), and porcine epidemic diarrhea virus (PEDV) have been reported to cause the cleavage of TAB1 or NEMO through their individual 3CLpro (Table 1, yellow highlighted row), suggesting an important role for 3CLpro in antagonizing the host antiviral innate immune response.

The importance of virus-induced cytoplasmic aggregates, termed antiviral stress granules (avSGs), in RLRs-mediated innate immunity has been increasingly recognized. Upon viral infection, dsRNAs are generated and accumulate in the cytoplasm, which activate the viral RNA sensor protein kinase R (PKR) and cause phosphorylation of eukaryotic initiation factor 2α and consequent formation of avSGs. Together with key molecules (i.e., Ras-GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) and T cell intracellular antigen 1), avSGs are formed by recruiting multiple antiviral effectors, such as RLRs, Pumilio, DEAH-box helicase 36, Mex-3 RNA binding family member C, tripartite motif containing 25, OAS and RNaseL. The close-proximity of 5’-triphosphate containing ssRNA and dsRNA with RLRs within the compact avSG compartment facilitates a more robust RLR-mediated IFN-I responses to suppress viral replication. To bypass this, picornaviruses, including PV, EMCV, CBV3, FMDV, and EV-D68, utilize 3Cpro to cleave G3BP1 and block avSG formation to prolong viral survival.

CoVs were also found to be able to modulate the formation of avSGs. It was reported that MERS-CoV accessory protein 4a prevents PKR activation by directly binding viral dsRNA, thereby inhibiting avSG formation allowing for effective viral replication. However, the role of protein 4a in avSG formation...
Picornaviruses evade type I interferon immune response via the function of 3C protease (3Cpro). Binding of picornaviruses to their respective receptors facilitates their entry into the cells and release of the 5'-viral protein genome-linked-containing genomic RNA into cytoplasm. Long double-stranded RNA generated during the replication process binds to MDA5, exposing its CARD and allowing homotypic CARD-CARD interactions with its downstream adapter, MAVS. Subsequently, MAVS triggers the expression of IFN-I genes (Ifnb1 and Ifna in dendritic cells) and ISGs for antiviral purposes through the activation of transcription factor IRF3/7 and NF-κB. To facilitate a robust signaling, more efficient detection of dsRNA occurs in antiviral stress granules. Targets of viral encoded 3Cpro are indicated. CARD, caspase activation and recruitment domain; CTD, C-terminal binding domain; G3BP1, Ras GTPase-activating protein-binding protein 1; IFN-I, type-I interferon; IKKe, inhibitor of nuclear factor-κB (IκB)-kinase-ε; IRF3/7, interferon regulatory factors-3/7; ISGs, interferon-stimulating genes; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma-differentiation associated protein-5; NF-κB, nuclear factor-κB; NEMO, NF-κB essential modulator; P, phosphate-group; RIG-I, retinoic acid-inducible gene-I; TBK1, TANK binding kinase-1; TLR3, Toll-like receptor 3; TRAF3, TNF-receptor associated factor-3; TRIF, Toll/IL-1 receptor domain-containing adapter-inducing interferon-β.
| Proteinase | Virus                      | Gene symbol | Full gene name                                      | Cleavage site | Implication to host                                                                 | Ref       |
|------------|---------------------------|-------------|----------------------------------------------------|---------------|-------------------------------------------------------------------------------------|-----------|
| **Host defense and inflammation** |                           |             |                                                    |               |                                                                                     |           |
| 3C         | CVB3, RV, EV-A71, SVW     | RIG-I       | Retinoic-acid-inducible gene-1                     | N/A           | Dampered cytokines production; enhanced viral propagation                           | 33,39     |
| 2A         | PV, EV-A71, CVB3          | MDA5        | Melanoma differentiation-associated protein 5      | N/A           |                                                                                     | 37.40     |
| 2A 3C, 3CLSP | CVB3, HRV, EV-A71, PV, SVW, PRRSV | MAV5       | Mitochondrial antiviral signaling                  | Q148↓, Q209↓, Q251↓, Q265↓, Q269↓ |                                                                                     | 34,36,38,40,41,46 |
| 3C         | EV-D68, EV-A71, CVB3, HAV, SVV | TRIF       | TIR domain-containing adapter inducing interferon beta | Q159↓, Q190↓, Q312↓, Q554↓, Q653↓ |                                                                                     | 36.47,48  |
| PL         | SARS-CoV-2                | IRF3        | Interferon regulatory factor 3                     | G270↓         |                                                                                     | 49        |
| 3C         | EV-D68, EV-A71            | IRF7        | Interferon regulatory factor 7                      | Q167↓, Q189↓  |                                                                                     | 35.50     |
| 3C         | EV-A71                    | IRF9        | Interferon regulatory factor 9                      | N/A           |                                                                                     | 51        |
| 2A 3C       | EV-A71, CVB3              | NLRP3       | NACHT, LRR, and PYD-domain-containing protein 3    | Q225↓, Q226G↓, Q493↓ |                                                                                     | 52.53     |
| 3CL        | SARS-CoV-2                | NLRP12      | NACHT, LRR, and PYD-domain-containing protein 12   | Q238↓, Q293↓  |                                                                                     | 49        |
| 3C         | EMCV, SVV                 | TANK1       | TRAF family-member-associated NF-kB activator-1    | E272 and Q291 |                                                                                     | 54        |
| 2A         | EV71                      | IFNAR       | Interferon alpha and beta receptor subunit 1       | N/A           |                                                                                     | 55        |
| 3C         | HRV, PV                   | C3          | Complement C3                                      | N/A           |                                                                                     | 56        |
| **3C 3CL** | FMDV, PDCoV               | STAT2       | Signal transducer and activator of transcription 2 | Q685↓, Q758↓  |                                                                                     | 43,44,57–61 |
| 3C 3CL, 3CLS | FMDV, HAV, PEDV, PRRSV  | NEMO        | NF-kB essential modulator                          | Q304↓, Q383↓, Q231↓, Q349↓ |                                                                                     | 55        |
| **3C**     | EV-D68, EV-A71, CVA6, CVA16 | TAK1       | Transforming growth factor-β-activated kinase 1    | Q360↓, Q361↓  |                                                                                     | 37.45,49  |
| 3C 3CL     | EV-A71, SARS-CoV-2        | TAB1        | TAK1 binding protein 1                             | Q414↓, Q451G, Q451j, Q452j, Q132↓, Q133↓, Q444↓ |                                                                                     |           |
| 3C         | EV-A71                    | TAB2        | TAK1 binding protein 2                             | Q113↓, Q114S  |                                                                                     |           |
| 3C         | EV-A71                    | TAB3        | TAK1 binding protein 3                             | Q173↓, Q174G, Q343↓, Q344G |                                                                                     |           |
| 3C         | CVB3, PV                  | RIP1, RIP3  | Receptor interacting protein-1, -3                 | Q134↓, Q430↓, R118↓, R119↓ |                                                                                     | 62,63     |
| 3C         | EV-A71                    | ZAP         | Zinc-finger antiviral protein                       | Q360↓, Q370G  |                                                                                     | 64        |
| 3C         | EV-A71                    | GSDMD       | Gasdermin-D                                        | Q193↓, Q194G  | Inhibition of pyroptosis & promotion of viral growth                                 | 65        |
| **Autophagy** |                           |             |                                                    |               |                                                                                     |           |
| 2A         | CVB3                      | p62/SQSTM1  | Sequestosome-1                                     | Q241↓         | Disruption of cellular autophagy pathway                                            | 66–70     |
| 2A         | CVB3                      | NBR1        | Neighbor of BRCA1 gene                             | G402↓, E682↓  |                                                                                     |           |
| 3C         | CVB3                      | CALCOCO2/NDP52 | Calcium binding and coiled-coil domain containing protein 2; Nuclear dot 10 protein 52 | Q139 |                                                                                     |           |
| 3C         | CVB3                      | SNAP29      | Synaptosomal-associated protein 29                  | Q161↓         |                                                                                     |           |
| 3C         | CVB3                      | PLEKHM1     | Pleckstrin homology domain and RUN domain Containing M1 | Q668↓ |                                                                                     |           |
| Proteinase | Virus          | Gene symbol | Full gene name                      | Cleavage site | Implication to host                                                                 | Ref  |
|------------|----------------|-------------|-------------------------------------|---------------|-------------------------------------------------------------------------------------|------|
| Cellular Integrity | 2A   | CVB3        | DYSF                               | N/A           | Disruption of muscle membrane repair                                                | 71   |
|             | 2A   | CVB3, CVB4  | DMD                                | Human: (G589↓; G2,435↓) Mouse: (G591↓; G2,435↓) | Muscular dystrophy, especially heart muscle                                         | 72   |
|             | 2A   | HRV2        | CK-II                              | G15↓          | Disruption of cytoplasmic cytoskeleton                                             | 73   |
|             | 3C   | PV          | MAP-4                              | Q188↓,189G    | Collapse of microtubules, disruption of host protein dynamics and interactions      | 74   |

| Transcription | 3C   | PV          | Oct-1                              | Q330↓,331G    | Disruption of polymerase activity, affecting gene transcription                    | 75   |
|               | 3C   | PV          | La/SSB                             | Q358↓,359G    | Disruption of SRF-dependent gene expression (e.g. cardiac contractile and regulatory factors) | 76   |
|               | 2A   | PV          | TBP                                | Q18↓,19G, Q104↓,1055 | Disruption of normal RNA metabolism (e.g. splicing)  | 77–80 |
|               | 3C   | PV          | TFIIIA/C                           | N/A           |                                      | 81,82 |
|               | 3C   | PV          | CREB                               | Q172↓,173G    |                                      | 83   |
|               | 2A   | CVB3        | SRF                                | G327          |                                      | 84   |
|               | 3C   | PV          | PTB/p52/ hnrNAP-I                  | Q148↓,152/321A |                                      | 85   |
|               | 3C   | PV          | hnrNRP M, K                        | Q389↓,390E, Q364↓365G |                                      | 86   |
|               | 2A   | PV          | Gemin3                             | G463↓         |                                      | 87   |
|               | 3C   | CVB3        | TDP-43                             | Q327↓,328A    |                                      | 88   |
|               | 3C   | PV          | p65-RelA                           | Q480↓,481G    |                                      | (Continues) |
| Proteinase | Virus | Gene symbol | Full gene name | Cleavage site | Implication to host | Ref |
|-----------|-------|-------------|----------------|--------------|---------------------|----|
| Translation and RNA turn over | 3C | CVB3 | AUF1 | AU-rich element RNA-binding factor 1 | N/A | Disruption of host mRNA turn over | 89 |
| 2A, 3C | PV, CVB3, HAV, EMCV, FMDV, DHA V | PA BP | Poly(A)-Binding protein | Q537, Q367, Q368G, M487, G488, Q437, Q438G | Host protein translation shut-off, disruption of SG formation | 90–93 |
| 3C | FMDV | eIF4A | Eukaryotic elongation factor 4A | Ge74, 675R, R481, G482 | | 94–99 |
| L, 2A, 3C | FMDV, PV, CVB3, EV-A71, RV | eIF4G | Eukaryotic elongation factor 4G-1/4G-2 | Ge74, 675R, R481, G482 | | 94–99 |
| 3C | PV, CVB3, HRV | eIF5S | Eukaryotic elongation factor 5S | Q478, G488 | ↓ | 100 |
| 2A | CVB3 | DAP5 | Death-associated protein 5 | G434 | ↓ | 101 |
| 3C | PV | PCBP2 | Poly(C)-Binding protein-2 | Q253, Q306, S254 | ↓ | 102 |
| 3C | PV | DCP1a | mRNA-decapping enzyme 1A | N/A | | 103 |
| Others | 3C | CVB3 | IKBa | Inhibitor of κBα | Q249, 250G | Constitutive activation of NFκB α and apoptosis | 104 |
| 2A, 3C | CVB3 | GAB1 | Growth factor receptor bound 2-associated binding protein-1 | G175, G436, G238 | | 105 |
| 2A | HRV, PV | Nup62, Nup98, Nup153 | Nucleoporins | A1031, G1771, G2011, G2181, G2471, A2981 | | 106, 107 |
| L, 3C | CVB3, PV, EMCV, FMDV, ERA V | G3BP1, G3BP2 | Ras-GTPase activating SH3-binding protein-1 | Q325, E284, Q325 | | 108–113 |
| 3C | CVB3, PV | GBF1 | Golgi-specific brefeldin A-resistance guanine exchange factor-1 | Q1,297, 1298 | | 63 |
| | | ACLY | ATP citrate lyase/synthase | Q777, 778A | | 63 |
| | | p115/USO1 | General vesicular transport factor p115 | Q832, 833G | | | |
| | | ALIX/PDCD6IP | Programmed cell death 6-interacting protein | Q728, 729S | | 108–113 |
| | | PFAS | Phosphoribosylformylglycinamidine synthetase | Q472, 473G | | 108–113 |

Abbreviations: CVA6/A16/B3, Coxsackievirus A6/A16/B3; CALCOCO2, coiled-coil domain-containing protein 2; DHA V, duck hepatitis A virus; EMCV, encephalomyocarditis virus; ERA V, equine rhinitis A virus; FMDV, foot-and-mouth disease virus; GBF1, golgi-specific brefeldin A-resistance guanine nucleotide exchange factor; GSDMM, gasdermin D; HAV, hepatitis A virus; HRV, human rhinovirus; hnRNP M/K, heterogeneous nuclear ribonucleoprotein M/K; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein 5; NDP52, nuclear dot 10 protein 52; NBR1, neighbor of BRCA1; NLRP3, NLR family pyrin domain containing protein 3; NEMO, NF-κB essential modulator; NFXB, nuclear factor-κB; PDCoV, porcine deltacoronavirus; PEDV, porcine epidemic diarrhea virus; PFAS, phosphoribosylformylglycinamidine synthetase; PLEKH1, pleckstrin homology domain and RUN domain containing M1; PRRs, pathogen recognition receptors; PRRSV, porcine reproductive and respiratory syndrome virus; RIP1, receptor-interacting serine/threonine-protein kinase 1; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; SSV, Seneca Valley virus; STAT2, signal transducer and activator of transcription 2; TAB1, TAK1 binding protein-1; TRIF, (TIR)-domain-containing adapter-inducing interferon-β

Note: Color coding correspond between virus and cleavage site
appears to be cell type-specific. MERS-CoV mutant with deletion of 4a gene still impedes avSG formation in certain cell types, suggesting that additional proteins (possibly viral proteases) encoded by MERS-CoV are required to antagonize avSGs. In a recent report, Grogan and colleagues utilized an affinity purification mass spectrometry proteomics approach to screen for cellular proteins that interact with individual SARS-CoV-2 proteins, including 3CLpro. Using both wild-type and catalytically inactive (C145A) 3CLpro, they identified two high-confidence interactions with the histone deacetylase 2 (HDAC2) and tRNA methyltransferase 1 (TRMT1), respectively. Of interest, HDAC2 has been previously shown to be required for IFN-I signaling through histone modification. Remarkably, in addition to 3CLpro, other SARS-CoV-2-encoded proteins were also found to interact with cellular proteins involved in regulating host innate immunity, including the core avSG protein G3BP1, a known antiviral protein that induces the innate immune response.

Clinical evidence revealed that SARS-CoV-2 infections predispose patients to subsequent bacterial or other viral infections, which are associated with poor outcomes including increased severity and fatality. Based on what is known about the role of 3Cpro during picornaviral infections, at least two mechanisms may contribute to the secondary infections. First, TRIF is not only involved in antiviral but also participates in antibacterial host defense mechanisms. Disruption of TRIF during the initial phase of viral infection could render the host more susceptible to bacterial invasions. Second, recent studies identified G3BP1 as a critical component in cyclic GMP-AMP synthase (cGAS)-mediated innate immune responses against DNA viruses. cGAS is a cytosolic DNA sensor that detects viral DNA to activate the IFN-I pathway. Cleavage of G3BP1 is thus expected to weaken this mechanism and leave the patients at higher risk from a secondary DNA viral infection. A proposed model that picornaviruses (probably CoVs as well) induce two phases of infection (or co-infection) and disease progression is depicted in Figure 3.

2.2 Targeting NLR family PYD containing protein-3 inflammasome pathway

Similar to RLRs, NLRs are cytosolic PRRs that sense intracellular PAMPs and/or danger-associated molecular patterns (DAMPs) to induce pro-inflammatory cytokine production and inflammatory cell death (termed pyroptosis). Under stress, NLRs initiate the formation of a large multiprotein complex, called inflammasome. The best-studied inflammasome is the NLR family PYD containing protein-3 (NLRP3) inflammasome, which consists of the sensor NLRP3, the adapter apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and the effector caspase-1. Upon sensing viral PAMPs and DAMPs generated during infection, NLRP3 oligomerizes and recruits pro-caspase-1 through the adapter protein ASC to form NLRP3 inflammasome. Subsequently, pro-caspase-1 undergoes self-cleavage and activation, promoting maturation and secretion of pro-inflammatory cytokine interleukin-1β (IL-1β) and IL-18, and inducing cleavage of the pro-pyroptotic factor gasdermin D (GSDMD). The resulting N-terminal cleavage product of GSDMD then creates pores on cell membrane, triggering pyroptotic cell death and facilitating release of inflammatory cytokines (IL-1β and IL-18). Similar to other types of cell death, pyroptosis limits viral replication by eliminating infected cells.

As NLRP3 plays a vital role in antiviral response, numerous viruses, including picornaviruses, have adopted strategies to counteract its functions. It was reported that NLRP3-, ASC-, and caspase-1-deficient mice infected with EV-A71 display more severe disease phenotype and higher virus titers as compared to wild-type control mice, suggesting a defensive role for the NLRP3 inflammasome against EV-A71 infection. Further investigation identified that, to overcome the antiviral immune response, EV-A71 has evolved to inactivate the NLRP3 inflammasome by directly cleaving NLRP3 through the proteolytic activities of 2Apro and 3Cpro. In addition, EV-A71 3Cpro also targets its effector GSDMD for cleavage at a site (Gln193-Gly194) different from that (Asp275-Asp276) mediated by caspase-1. The cleavage products of GSDMD by 3Cpro fail to stimulate pyroptosis; hence, further enhancing EV-A71 replication. Similarly, following CVB3 infection, NLRP3-/- mice present more rapid disease progression and increased viral loads when compared with wild-type mice. To evade the host defense, CVB3 3Cpro directly targets NLRP3 and its upstream signaling molecules (RIP1/RIP3, receptor-interacting protein 1/3) for degradation.
It is increasingly recognized that early, temporary activation of NLRP3 inflammation has an antiviral role by clearing virus and infected cells, whereas prolonged and extreme activation is harmful, causing disease-related immunopathology. For example, during hepatitis C viral infection, activation of NLRP3 inflammasome is associated with enhanced inflammation and tissue damage. Persistent activation of NLRP3 during CVB3 infection has also been linked to pathological outcome. It is well documented that SARS-CoV-2 infection leads to cytokine storm, featured by excessive production and secretion of pro-inflammatory cytokines and chemokines, contributing significantly to disease severity of Covid-19. Several proteins encoded by SARS-CoV, including E, ORF3a, and ORF8b, have been shown to activate the NLRP3 inflammasome. Recent evidence has also revealed that NLRP3 inflammasome is activated in response to SARS-CoV-2 infection. It is speculated that overactivation of the inflammasome may be responsible, at least in part, for the observed cytokine storm in Covid-19 patients.

The NLRP inflammasome pathway and viral manipulation are summarized in Figure 4.

2.3 Targeting host RNA degradation components

In eukaryotic cells, posttranscriptional processes (e.g., mRNA surveillance, silencing, translational repression, and degradation) play a central role in the regulation of gene expression and ultimately determine the expression levels of a significant fraction of the transcriptome. Recently, it has become apparent that posttranscriptional processes acting on cytoplasmic messenger ribonucleoprotein complexes (mRNPs) are physically tied and can occur in discrete cytoplasmic entity known as processing (P)-bodies. These compartments are highly conserved across cells derived from vertebrates, invertebrates, yeasts and plants, containing many enzymes involved in mRNA turnover. To date, P-bodies have been demonstrated to play critical roles in general mRNA degradation, nonsense-mediated

![Figure 4: Picornaviral 3CPro targets the NLRP3 inflammasome for immune evasion. RNA viruses and other DAMPs could activate NLRP3 inflammasome. GSDMD is also cleaved by caspase 1 and the resulting N-terminal cleavage products are inserted into the plasma membrane, forming multiple pores and inducing pyroptosis and release of pro-inflammatory cytokine. Upon picornaviral infection, NLRP3, its upstream signaling proteins (RIP1/RIP3), and its downstream effector GSDMD are all targeted by 3CPro for degradation. As a result, pyroptosis is inhibited for efficient viral replication. CARD, caspase activation and recruitment domain; DAMPs, danger-associated molecular patterns; GSDMD, Gasdermin D; LRR, Leucine rich repeat; NLRP3, NLR family and pyrin domain-containing protein 3; PYD, Pyrin domain; RIP, receptor-interacting protein; NACHT, NAIP, CIITA, HET-E and TEP1-associated families; VPg, viral protein genome-linked; 3CPro, 3C protease]
mRNA decay, adenylate-uridylate-rich (AU-rich) element-mediated mRNA degradation at the 3' UTRs, and miRNA-induced RNA silencing pathway.\textsuperscript{157}

In eukaryotes, the degradation process is initiated firstly by removal of the poly(A) tail by deadenylases. Following deadenylation, mRNAs are subjected to exonucleolytically degradation starting from their 3'-end by the exosome, a multimeric complex with 3'→5' exonucleases. In the meantime, the guanosine cap structure at the 5'-end is removed by several decapping enzymes and coactivators, including decapping protein 1/2 (DCP1/2), DEAD-box RNA-helicase-6 (DDX6), enhancer of mRNA decapping protein-3 (EDC3) and EDC4, rendering the mRNA susceptible to 5'→3' degradation by the major cytoplasmic exoribonuclease 1 (XRN1).\textsuperscript{158}

RNA degradation has emerged as an important antiviral host defense mechanism.\textsuperscript{158} The most widely studied viruses in the context of XRN1 are positive-stranded RNA viruses from the Flaviviridae family, including Dengue virus, West Nile viruses, hepatitis C

**FIGURE 5** Dispersion of 5'→3' RNA degradation components within P-bodies during picornaviral infection. RNA viruses, including picornaviruses, initiate viral replication in a discrete compartment within cytoplasm, generating various RNA species with defined signatures. These includes 5'ppp, 5'-ssRNA, dsRNA and viral mRNA. Both picornavirus and coronavirus are able to generate long ssRNA and dsRNA and trigger the translocation of 5'→3' RNA degradation components, including DCP1, DCP2, and XRN1, into the viral replication complex for degradation of associated viral RNA species. Picornaviral 3C\textsuperscript{pro} cleaves DCP1, resulting in increased viral particles and infectivity. DCP1/2, decapping protein-1/2; XRN1, 5'→3' exoribonuclease-1; dsRNA, double-stranded RNA; 3C\textsuperscript{pro}, 3C protease
virus, and Yellow Fever virus. XRN1 acts as an antiviral factor by degrading their genomic RNA. In addition, DCP1/2-XRN1 were shown to have critical antiviral functions against both picornaviruses and negative-stranded cytoplasmic RNA viruses. During infection, these viruses generate various RNA intermediate species with defined structures (long/short dsRNA and ssRNA) inside their replication complexes. Both long ssRNA and dsRNA could induce the re-localization of both DCP1/2 and XRN1 nucleases into the viral replication complexes for viral RNA degradation. This further supports the prior observation that the presence of XRN1 and DCPs potentially poses a threat to enterovirus RNA. To conquer this antiviral activity, PV utilizes its 3Cpro to disperse and destabilize P-bodies by directly targeting DCP1a for degradation. The interplay between the cellular RNA degradation pathway and the viruses is illustrated in Figure 5.

While both the genomic and subgenomic SARS-CoV-2 mRNAs contain 5′-cap structure, previous studies have shown that inhibiting several eukaryotic initiation factors family proteins (eIF4E, eIF4F, and eIF4G) could impair coronavirus replication, highlighting the importance of cap-dependent translation in SARS-CoV-2 mRNA synthesis. Thus, it is not surprising that SARS-CoV-2 would intervene the function of host decapping enzyme DCP1/2 and XRN1, possibly through its 3Cpro. However, to date, antiviral roles XRN1 in coronavirus mRNA translation have not been reported.

### 2.4 Targeting host autophagy machinery

Macroautophagy (or autophagy in short) is a conserved intracellular degradation pathway that is essential in maintaining cellular homeostasis by removing unwanted or dysfunctional cellular components. The process of autophagy is highly regulated by more than 30 “autophagy-related” proteins and includes three major steps. First, the substrates are sequestered by a crescent-shaped double-membrane vesicle called a phagophore. Then, the two ends of the phagophore fuse to form an autophagosome. Finally, autophagosome fuses with a lysosome while the enwrapped cargo is degraded by hydrolysis.

**Figure 6** Subversion of host autophagy through picornaviral 3Cpro. Schematic diagram depicted the molecular mechanism for the initiation of host autophagy upon the presence of RNA virus for the clearance of viral-associated molecules. Picornaviruses utilize its own encoded 2Apro (not shown here) and 3Cpro to cleave key components such as p62, NBR1, SNAP29, and PLEKHM1 to facilitate a more robust replication. ATGs, autophagy-related genes; DFCP1, double FYVE-containing protein-1; FIP300, focal adhesion kinase family interacting protein of 200kD; NBR1, neighbor of BRCA1; SNAP29, synaptosomal-associated protein-29; PLEKHM1, Pleckstrin homology and RUN domain containing M1; p62, also known as sequestosome 1 (SQSTM1); STX17, Syntaxin 17; ULK, Unc-51-like kinase-1; UVRAG, UV radiation resistance-associated gene protein; VAMP8, vesicle-associated membrane protein-8; WIPI2, WD-repeat domain phosphoinositide-interacting protein-2; 2Apro, 2A-protease; 3Cpro, 3C protease.
Autophagy plays a significant role in antiviral host defense by directly targeting invading viruses through a process, called virophagy, for clearance. Virophagy is mediated through the function of autophagy cargo receptors, including sequestosome 1 (SQSTM1)/p62, neighbor of BRCA1 (NBR1), calcium binding and coiled-coil domain-containing protein 2 (CALCOCO2)/nuclear dot 10 protein 52 (NDP52), which recruit viral components/particles to autophagosome for degradation. To evade the antiviral efforts of virophagy, many viruses, including picornaviruses, have evolved to disrupt the function of autophagy receptors. For instance, SQSTM1/p62 and CALCOCO2/NDP52 are targeted for degradation by CVB3 2Apro and 3Cpro, respectively. Cleavage of SQSTM1/p62 was later confirmed upon PV, HRV-1A, and EV-D68 infection. Furthermore, NBR1, a homolog of SQSTM1/p62, can also be cleaved by 3Cpro. Remarkably, it was found that cleavage of SQSTM1/p62 and NBR1 not only causes a loss-of-function, but also generates dominant-negative mutants against the function of native proteins. Loss of both SQSTM1/p62 and NBR1 would also impair mitophagy and results in mass production of reactive oxygen species, and IL-1β through constitutive NLRP3-independent inflammasome activation (potentially other NLRP family members). The resulting cleavage fragments of SQSTM1/p62 and NBR1 will accumulate to serve as DAMPs signals and further amplify the inflammatory cascade. Acute respiratory pneumonia due to cytokine storm is a hallmark of SARS-CoV-2 infection, whether its 3Cpro would manipulate host autophagic system in particular to mass produced IL-1β in Covid-19 pathogenesis is certainly worth further investigation.

**FIGURE 7** Schematic workflow of TAILS N-terminomics screening of 3Cpro and 3CLpro substrates. Schematic diagram depicts the TAILS workflow and scheme for identification of 3Cpro or 3CLpro substrates. In brief, protein samples from whole cell lysates were subjected to in vitro cleavage by either recombinant purified WT 3Cpro/3CLpro or mutant (C147A) 3Cpro/(C145A) 3CLpro, followed by N-terminal enrichment using TAILS (left panel). Samples were then combined and subjected to pre-TAILS shotgun-like mass-spectrometry analysis after complete digestion with trypsin. The exposed amine groups of N-terminals generated by the trypsin digestion were then removed by covalently coupling to a high-molecular weight polyaldehyde polyglycerol polymer. This process allowed for selection via negative enrichment of blocked N termini (middle panel). Peptides were subsequently identified and quantified using high-resolution mass spectrometry (indicated in the right panel). The resultant high-confidence candidate substrates were determined through the analysis of the quantified heavy/light (H/L) ratio of dimethylation-labeled semitryptic neo-N terminus peptides. They will be subjected to further validation through similar in vitro cleavage assay by 3Cpro/3CLpro, followed by immunoblotting using specific antibodies; TAILS, terminal amine isotopic labeling of substrates; 3Cpro, 3C protease; 3CLpro, 3C-like protease
| Research phase | Compound | Target | Results | Ref |
|----------------|----------|--------|---------|-----|
| In vitro validation | TG-0205221 | 3C\textsuperscript{pro} of SARS-CoV and HCoV-229E | Reduces SARS-CoV and HCoV-229E replication by titer of 4.7 Log\textsubscript{10} | 191 |
| | Flavonoids: Apigenin, luteolin, quercetin, amentoflavone, aueretin, daidzein, pueraerin, epigallocatechin gallate, gallocatechin gallate, kaempferol/hoirolin, pectolinarin, herbacetin, flavonol | SARS-CoV 3C\textsuperscript{pro} | Inhibit SARS-CoV 3CL\textsuperscript{pro} FRET protease assay catalytic activity | 192 |
| | Pyrazolone and pyrimidines inhibitors | SARS-CoV 3C\textsuperscript{pro} | Show potent inhibitory activities against SARS-CoV 3CL\textsuperscript{pro} at micromolar range. | 193 |
| | Aryl methylene ketones, Mono-, and difluorinated methylene ketones groups | SARS-CoV 3C\textsuperscript{pro} | Improved version is stable and less toxic to cells. Potently inhibits SARS-CoV 3CL\textsuperscript{pro} at nanomolar range. | 194 |
| | Heteroaromatic esters and benzotriazole esters derivatives | SARS-CoV 3C\textsuperscript{pro} | Show potent inhibitory activities against SARS-CoV 3CL\textsuperscript{pro} at nanomolar range. | 195-197 |
| | Boronic | SARS-CoV 3C\textsuperscript{pro} | Significantly inhibits SARS-CoV 3CL\textsuperscript{pro} enzymatic activity in micromolar range. | 198 |
| | Aza-peptide epoxides derivatives | SARS-CoV 3C\textsuperscript{pro} | Show irreversible inhibition against SARS-CoV 3CL\textsuperscript{pro}. | 199-201 |
| | Etacrynic acid derivatives | SARS-CoV PL\textsuperscript{pro} and 3C\textsuperscript{pro} | Show more than 70% inhibition on SARS-CoV at concentration of 100μM | 202-203 |
| | Peptides aldehydes derivatives | SARS-CoV and HCoV-229E 3C\textsuperscript{pro} | Suppress SARS-CoV by 4.7 Log\textsubscript{10} and HCoV-229E by 5.2 Log\textsubscript{10} | 191 |
| | Modified version of HIV protease inhibitors | SARS-CoV 3C\textsuperscript{pro} | Potent inhibitors against SARS-CoV 3CL\textsuperscript{pro} but not against HIV protease | 204 |
| | Sulfone and dihydroimidazole derivatives | SARS-CoV 3C\textsuperscript{pro} | 21 derivatives from these two analogs show EC\textsubscript{50} less than 50 μM against SARS-CoV 3CL\textsuperscript{pro}. | 205-206 |
| | Michael acceptor peptidomimetics | SARS-CoV 3C\textsuperscript{pro} | Show potent inhibitory against SARS-CoV 3CL\textsuperscript{pro}. | 207-208 |
| | Lignoids, di- and triterpenoid derivatives: Betulinic acid, savinin, ferruginol, pritimererin, tingenne, iguestrin and triterpenoids celsanol | SARS-CoV 3C\textsuperscript{pro} | Abietane type diterpenoids are the most robust terpenoids on SARS-CoV (EC\textsubscript{50} = 9.1 μM) | 209-211 |
| | Metal conjugated: Zinc- or mercuric based | SARS-CoV PL\textsuperscript{pro} and 3C\textsuperscript{pro}, | Inhibition is pronounced in Zinc-conjugated compounds | 212-213 |
| | α-Ketoamides: | 3C\textsuperscript{pro} of CVB3, &; HRV, EV-D68, EV-A713CL\textsuperscript{pro} of SARS-CoV, MERS, 229E | Display low toxicity & low micromolar of EC\textsubscript{50} against tested viruses | 214 |
| | Pyridyl, pyrazyl and Benzotriazole-derivatives inhibitors | SARS-CoV PL\textsuperscript{pro} or 3C\textsuperscript{pro} | Robust inhibition on SARS-CoV in vitro within micromolar range | 215-217 |
| | | Rupintrivir (AG-7088) | Targeting 3C\textsuperscript{pro} and 3C\textsuperscript{pro} encoding viruses | Shows robust activity against SARS-CoV-2 in vitro | 218-219 |
| | | Boceprevir, Calpain inhibitors II, and XII | SARS-CoV-2 3C\textsuperscript{pro} | Inhibit SARS-CoV-2 in vitro with EC\textsubscript{50} less than 5μM | 220 |
| | | 3CL\textsuperscript{pro}-1 | Originally designed for 3C\textsuperscript{pro} of EV-A71. | Shows robust efficacy of EC\textsubscript{50} 200 nM, effective against SARS-CoV-2 and MERS-CoV. | 221-222 |
| | | Isatin derivatives | Targeting 3C\textsuperscript{pro} and SARS-CoV 3C\textsuperscript{pro} | Effectively inhibit SARS-CoV 3CL\textsuperscript{pro} through noncovalent bonding in low micromolar range | 223-224 |
| | | Anilide derivatives: 2-chloro-4-nitro aniline-, phenylalanine, 4-(dimethylamino)benzoic acid | SARS-CoV 3C\textsuperscript{pro} | Potent and highly specific inhibitors against SARS-CoV 3CL\textsuperscript{pro}. | 225 |
TABLE 2 (Continued)

| Research phase                      | Compound                                                                 | Target                  | Results                                                                                                   | Ref |
|-------------------------------------|--------------------------------------------------------------------------|-------------------------|-----------------------------------------------------------------------------------------------------------|-----|
| Computational prediction (docking analysis) | Anti-HIV-1 drugs: Indinavir, Darunavir                                    | SARS-CoV-2 3CL<sub>pro</sub> | Docking & binding free energy prediction shows high scores & high binding affinities against SARS-CoV-2 3CL<sub>pro</sub> | 226 |
| In vitro and in vivo validation     | Decahydroisoquinoline inhibitors                                         | SARS-CoV-2 3CL<sub>pro</sub> | X-ray crystallization studies confirmed that these inhibitors fit well into the cleft of 3CL<sub>pro</sub> | 227 |
|                                     | Peptides with halomethyl ketone derivatives                              | SARS-CoV 3CL<sub>pro</sub> | Effectively inhibit SARS-CoV infection, with low cytoxicity in cells and in mice.                       | 228 |
| Widely tested in animals and now under trial on human for coronavirus disease-2019 | GC376                                                                   | Targeting 3C<sub>pro</sub> and 3CL<sub>pro</sub> encoding viruses | Shows robust activity against SARS-CoV, SARS-CoV-2, and Norovirus                                      | 220 |

Abbreviations: HRV, human rhinovirus; MERS-CoV, Middle East respiratory syndrome-CoV; PLpro, papain-like protease; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2 3CL<sub>pro</sub>/3C<sub>pro</sub>; 3C-like protease/3C protease.

Decreased autophagic flux as a result of blockage of autophagosome-lysosome fusion is another mechanism adopted by picornaviruses to escape viral RNA/protein degradation. The fusion process is regulated by multiple proteins involved in membrane trafficking, particularly a group of SNAP29 proteins, including syntaxin 17 (STX17), synaptosomal-associated protein 29 (SNAP29), and vesicle-associated membrane protein 8 (VAMP8).<sup>168</sup> It was discovered that upon EV-D68 and CVB3 infection, the SNAP29 linker protein, SNAP29, is cleaved by 3C<sub>pro</sub>, which dissociates the STX17-interacting domain of SNAP29 from the VAMP8-binding motif, thereby disrupting the formation of STX17-SNAP29-VAMP8 complexes and inhibiting autophagosome-lysosome fusion.<sup>64,70</sup> Figure 6 summarizes the known mechanism by which picornaviral protease subverts the autophagy for immune evasion.

Similar to the observations made with several picornaviruses, it was recently demonstrated that SARS-CoV-2 infection impairs autophagic flux by blocking autophagosome fusion with a lysosome and induction of autophagy reduces viral replication.<sup>169</sup> This finding is consistent with an earlier report with MERS-CoV infection by the same research group.<sup>170</sup> Although the mechanism responsible for decreased autophagosome-lysosome fusion remains largely unclear, it was previously shown that over-expression of the membrane-associated PL<sub>pro</sub> of SARS-CoV and MERS-CoV is sufficient to suppress the fusion between autophagosome and lysosome.<sup>171</sup> Whether CoV 3CL<sub>pro</sub> has a role in regulation of autophagic flux and cargo recognition in general as some picornaviral 3C<sub>pro</sub> does has not been explored and requires further investigations. The list of substrates of 3C<sub>pro</sub> and 3CL<sub>pro</sub> is summarized in Table 1.

2.5 | High-throughput identification of cellular substrates of 3C<sub>pro</sub>

A proteomics-based quantitative method, termed N-terminomic terminal amine isotopic labeling of substrates (TAILS) (Figure 7),<sup>172</sup> has been exploited to globally search for novel cellular substrates of picornaviral proteases.<sup>63,85</sup> TAILS, developed by the Overall laboratory at the University of British Columbia, uses an unbiased negative selection approach to identify neo-N and C-termini (named N-terminomic and C-terminomic TAILS, respectively).<sup>172-175</sup> This state-of-the-art approach has several advantages, including quantitative, highly sensitive, and concurrent identification of both the substrates and the cleavage sites.<sup>176</sup> and has been used to analyze substrates of many types of proteases.<sup>177,178</sup>

Jan and colleagues conducted N-terminomic TAILS on HeLa cell or mouse cardiomyocyte extracts subjected to incubation with purified recombinant PV or CVB3 3C<sub>pro</sub>, respectively, to identify cleaved neo-N-terminal peptides (see Figure 7 for the detailed procedure).<sup>63,85</sup> A list of high confidence candidate proteins was generated for 3C<sub>pro</sub>, including peptides corresponding to previously reported substrates at the known cleavage sites (i.e., poly(A) binding protein [PABP]),<sup>70</sup> G3BP1<sup>110</sup> and TAR DNA-binding protein 43 (TDP-43)<sup>87</sup>. Among them, a subset of candidate targets of PV 3C<sub>pro</sub> has been validated in vitro and under viral infection, which include four common protein substrates of both PV and CVB3 3C<sub>pro</sub> (i.e., heterogeneous nuclear ribonucleoprotein M (hnRNPM), hnRNPK, RIP1, and phosphoribosylformylglycinamidine synthetase [PFAS]), programmed cell death 6-interacting protein (PDCD6IP, also known as ALIX), general vesicular transport factor p115, ATP-citrate synthase (ACLY), Golgi-specific brefeldin A resistance guanine nucleotide exchange factor 1 (GBF1). Studies through gene-silencing or expression of a non-cleavable mutant form of these substrates have demonstrated a pivotal role for these proteins in regulating viral replication/propagation.<sup>63,85</sup>

Given the effectiveness of the N-terminomic TAILS in identifying the cellular targets of 3C<sub>pro</sub>, this unbiased proteomics approach is currently being utilized to analyze the cellular targets of MHV and SARS-CoV-2 proteases, including 3CL<sub>pro</sub>. It is anticipated that identification of the full repertoire of host substrates of CoV proteases will provide a more comprehensive understanding of viral tropism,
interaction with host cells, and pathogenesis, as well as assist in the development of novel antiviral drugs.

2.6 | Current 3C\textsuperscript{pro} and 3CL\textsuperscript{pro} inhibitors and future challenges

As discussed earlier, both picornaviral 3C\textsuperscript{pro} and coronaviral 3CL\textsuperscript{pro} are chymotrypsin-like cysteine proteases with conserved substrate specificity (P1-P1’ and P4 cleavage sites of the substrates are highly conserved between two enzymes) and active sites.\textsuperscript{179} Owing to these similarities, efforts have been made to explore the potential of developing broad-spectrum antiviral compounds.\textsuperscript{13-15} The fact that no known human homologs further increases the feasibility of this strategy.

Rupintrivir (AG-7088, a protease inhibitor originally developed for HRV to treat common cold) and/or its derivatives/analogues have been found to possess antiviral activities against a wide range of picornaviruses (i.e., HRV, PV, EV-A71, EV-D68, CVB, CVA, HAV, and FMDV)\textsuperscript{180-183} and coronaviruses, including SARS-CoV.\textsuperscript{14,184} Furthermore, it was reported that dipeptidyl aldehyde (GC373), a-ketoamide (GC375), and dipeptidyl bisulfite adduct (GC376), are able to inhibit both picornaviral 3C\textsuperscript{pro} and coronaviral 3CL\textsuperscript{pro} activities and block viral replication.\textsuperscript{13} These studies point to the possibility of developing broad-spectrum antiviral therapeutics against SARS-CoV-2. However, rupintrivir failed to show efficacy in natural HRV infection conditions during a clinical phase II trial.\textsuperscript{185}

Although Remdesivir (a nucleotide analog inhibitor of RdRp, originally developed for the treatment of Ebola virus disease by Gilead Sciences) has been approved for treating severely ill Covid-19 patients in the United States and the Europe,\textsuperscript{186} its safety and antiviral activity are still under extensive investigations and clinical trials. Similarly, despite some early promising findings showing the effectiveness of chloroquine (a classical anti-malarial and autoimmune disease drugs) against SARS-CoV-2 infection in non-respiratory cells,\textsuperscript{187,188} recent research has demonstrated that chloroquine fails to block infection of human lung epithelial cells with SARS-CoV-2.\textsuperscript{189} In addition, it is known that chloroquine may cause severe cardiotoxicity.\textsuperscript{190} A number of attempts have been made to design drugs targeting 3CL\textsuperscript{pro} of SARS-CoV-2. Different approaches, including laboratory synthesis, virtual screening, drug repositioning, and structure-based molecular docking have been taken for this purpose. Hilgenfeld laboratory recently elucidated the X-ray crystallographic structure of SARS-CoV-2 3CL\textsuperscript{pro} and synthesized improved a-ketoamide inhibitors to target 3CL.\textsuperscript{12} Using computer-aided drug design, Jin et al. identified a 3CL\textsuperscript{pro} inhibitor (N3) and determined the crystal structure of its complex with 3CL\textsuperscript{pro} of SARS-CoV-2.\textsuperscript{10} Through a combined structure-based virtual and high-throughput screening, they further showed that six compounds exhibit potent anti-3CL\textsuperscript{pro} activities.\textsuperscript{10} Most recently, Dai et al. reported the design and synthesis of two lead compounds (11a and 11b) targeting 3CL\textsuperscript{pro} and solved the X-ray crystal structure of these inhibitors bound to 3CL.\textsuperscript{9} Although these compounds serve as promising drug candidates, their effectiveness in nature infection remain to be investigated. The list of inhibitors targeting 3C\textsuperscript{pro} and 3CL\textsuperscript{pro} is summarized in Table 2.

3 | CONCLUDING REMARKS

To date, we know very little about the 3CL\textsuperscript{pro} of SARS-CoV-2, especially the molecular mechanism of the pathways by which SARS-CoV-2 3CL\textsuperscript{pro} blocks. Certainly, we cannot rule out that there are some differences but also a lot of similarities among both families. While we are in the process of understanding the structure and functions of SARS-CoV-2 3CL\textsuperscript{pro}, a comparison with the 3C\textsuperscript{pro} from picornaviruses can provide more insights into the pathogenesis and regulatory mechanisms of Covid-19. These will serve as a critical foundation for the design of broad-spectrum anti-coronaviruses inhibitors, or perhaps anti-3C/3CL\textsuperscript{pro} expressing viruses.

ACKNOWLEDGEMENT

We thank all the members in Luo lab for insights and discussion. We apologize for not including all related references due to space constraints. C.S.N. is funded by VIROGIN Biotech Ltd. through the MITACS-Accelerate Program. This work was supported by grants from the Natural Sciences and Engineering Research Council (RGPIN2016-03811), the Canadian Institutes of Health Research (PJT-159546 and PJT-173318), and the Heart & Stroke Foundation of Canada (G-16-00013800 and G-18-0022051).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTIONS

Chen Seng Ng and Honglin Luo designed and outlined the review. Chen Seng Ng prepared the initial manuscript and figures. Christopher C. Stobart prepared the structural figure and relevant information. All authors contributed to revising and writing the final version.

DATA AVAILABILITY STATEMENT

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

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How to cite this article: Ng CS, Stobart CC, Luo H. Innate immune evasion mediated by picornaviral 3C protease: Possible lessons for coronaviral 3C-like protease? *Rev Med Virol.* 2021;31(5):e2206. doi:10.1002/rmv.2206