UJEMI PERSPECTIVES

3CLpro: The discovery of host cell substrates and its relevance as a drug target for SARS-CoV-2 variants of concern

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SUMMARY The virally-encoded proteases 3CLpro and PLpro are essential for SARS-CoV-2 replication. Since the onset of COVID-19, 3CLpro has been considered as a desirable drug target due to its conserved structure and function amongst coronaviruses and SARS-CoV-2 variants. 3CLpro and PLpro are known to cleave viral polyproteins post-translation, yielding non-structural proteins with different roles in the SARS-CoV-2 life cycle. However, recent studies have demonstrated that 3CLpro proteolytic activity is not only limited to viral proteins. 3CLpro has been shown to cleave an extensive range of host cell substrates, thereby subverting host antiviral responses to infection. Terminal Amine Isotopic Labelling of Substrates (TAILS) has identified at least 101 host cell proteins that are cleaved by 3CLpro during infection. Many of these substrates are involved in relevant biological pathways, including type I interferon signaling, cell differentiation and morphology, and autophagy. The implications for the host's antiviral response and therapeutic drug development remain poorly understood. In addition, 3CLpro in SARS-CoV-2 variants of concern harbors point mutations, potentially undermining its suitability as a therapeutic drug target. This article will provide a comprehensive review of 1) the recent discovery of the 3CLpro host cell degradome and how it subverts antiviral responses, and 2) whether mutations in 3CLpro compromise the efficacy of 3CLpro inhibitors, including PAXLOVID™, to treat SARS-CoV-2 variants of concern. Given this new insight on the mechanism of 3CLpro, we must understand why this protease requires so many host targets to hijack the immune system. The mutations and multifaceted function of 3CLpro are worth further investigation to justify the approval of therapeutic drugs targeting 3CLpro, and to reduce the clinical burden of the most concerning SARS-CoV-2 variants worldwide.

INTRODUCTION

Since the onset of the coronavirus disease 2019 (COVID-19) pandemic, hundreds of mutations have been recorded in the respiratory syndrome coronavirus 2 (SARS-CoV-2), leading to the emergence of variants of concern worldwide (1–4). The viral genome is estimated to evolve at a rate of one nucleotide substitution every 11 days, resulting in enhanced viral characteristics and pathogenicity, as shown by the alpha, beta, gamma and delta variants (1, 2). In particular, the recent B.1.1.529 variant (Omicron) harbors at least 30 mutations in the spike glycoprotein, 15 of which occur in the receptor-binding domain (RBD), leading to increased binding and entry into host cells (3, 5) and reduced efficacy of mRNA vaccines targeting this protein (4). Consequently, the Omicron variant has spread to over 80 countries (5) and currently poses a challenge to the development of new vaccines and therapeutics (4).

Unlike the heavily-mutated spike protein, the 3-chymotrypsin-like protease (3CLpro) of SARS-CoV-2 shares a highly conserved structure and function with other coronavirus homologs (6, 7). 3CLpro and papain-like protease (PLpro) are the two proteases encoded by SARS-CoV-2, and play a critical role in viral replication (Fig. 1). 3CLpro and PLpro are non-structural proteins (NSP) encoded in the positive-sense single-stranded RNA (+ssRNA) genome, which is translated into polyproteins 1a and 1ab by host cell ribosomes. The proteases, also known as nsp5 and nsp3, respectively, cleave themselves from the polyprotein by autoproteolysis (8). Subsequently, they cleave the remaining viral

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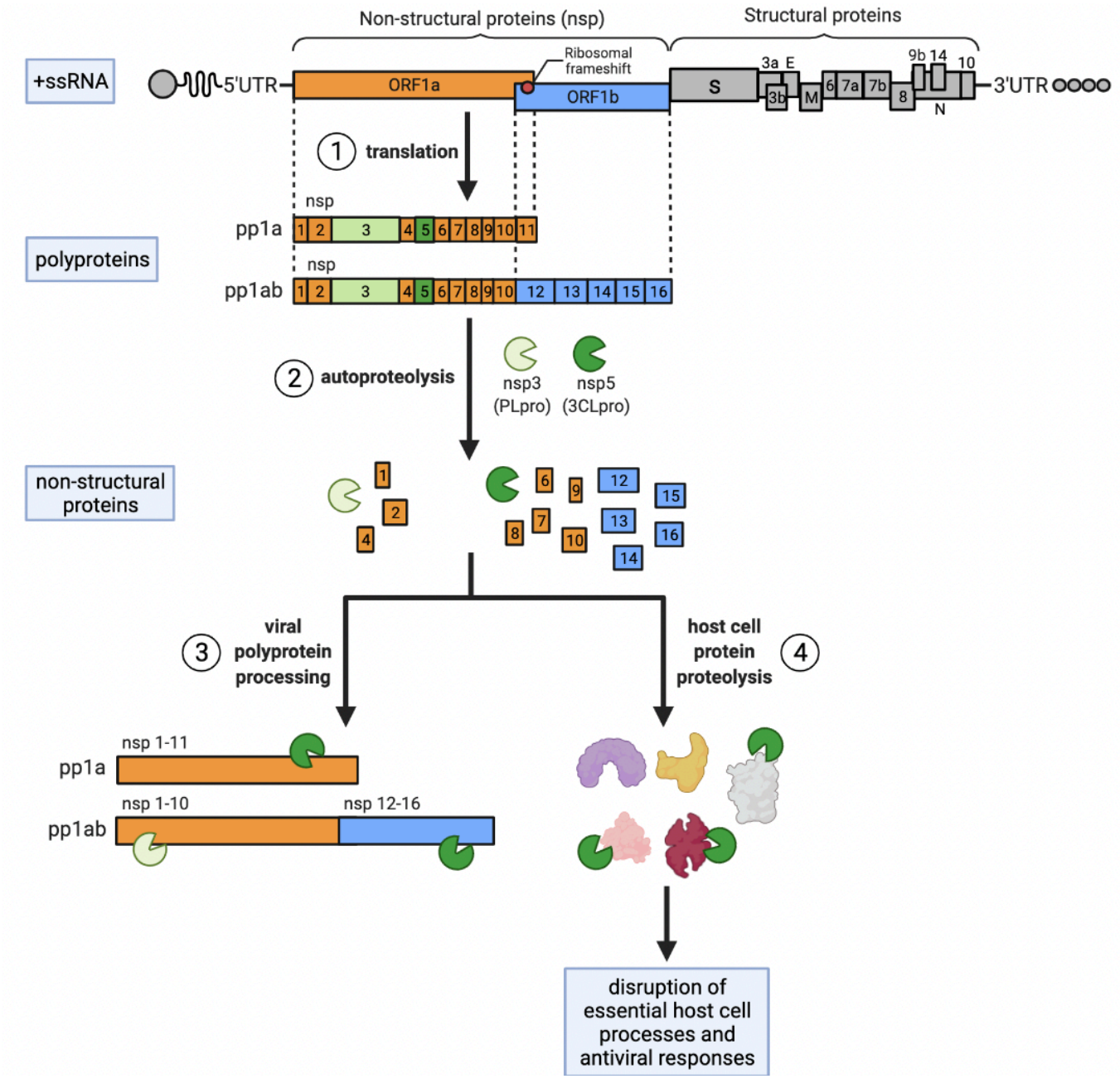

FIG. 1 Overview of SARS-CoV-2 polyprotein processing by 3CLpro and PLpro. (1) The viral +ssRNA genome is translated by host ribosomes. ORF1a/b = polyprotein (pp) open reading frames; S = spike, E = envelope; M = matrix; N = nucleocapsid; 3a, 3b, 6, 7a, 7b, 8, 9b, 14, 10 = accessory genes. (2) pp1a and pp1ab are autoproteolyzed by 3CLpro and PLpro, yielding nsp1-4 and nsp5-16, respectively. (3) RTC-associated 3CLpro and PLpro proteolyze other newly-translated pp1a and pp1ab. (4) 3CLpro cleaves host cell proteins involved in the antiviral response and essential cell pathways. Figure created with BioRender.com.

polyproteins to yield 16 NSPs with different functions in viral replication (8). Out of these 16 NSPs, the first 11 are cleaved by 3CLpro (9). After polyprotein processing, all of the NSPs hijack the endoplasmic reticulum (ER), where they form double-membrane vesicles and ER-associated replication and transcription complexes (RTC) (10) to further support SARS-CoV-2 replication.

Intriguingly, a recent study by Pablos *et al.* (11) has demonstrated that 3CLpro proteolytic activity is not only limited to viral polyproteins, but it also cleaves a wide range

of host cell proteins. Terminal Amine Isotopic Labelling of Substrates (TAILS) (12) was used to characterize the host cell degradome by selectively purifying the N-terminal peptides of SARS-CoV-2 3CLpro cleavage products from HEK293 and BEAS-2B cell lysates incubated with purified 3CLpro (Fig. 2). TAILS identified at least 101 host cell proteins that are irreversibly cleaved by 3CLpro with high specificity and fidelity (11). The authors validated the proteolysis of select host substrates *in vitro,* and attempted to characterize them to better understand their function within the context of host antiviral defenses. It is clear that the proteolytic repertoire of 3CLpro remains poorly understood and warrants further investigation.

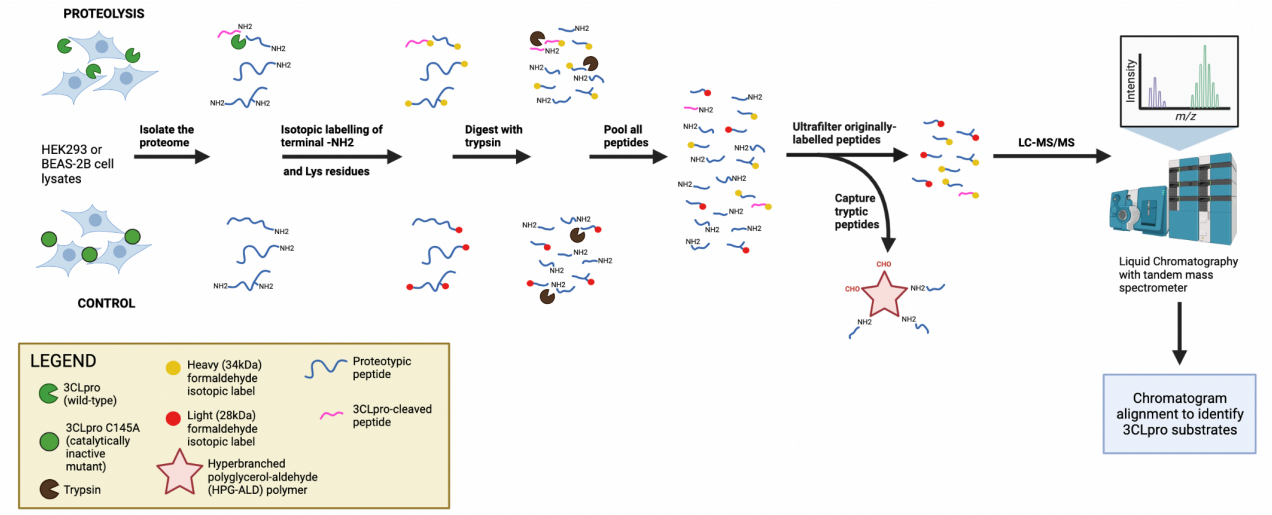

FIG. 2 Workflow of 3CLpro host cell degradome identification by TAILS. Left to right: HEK293 and BEAS-2B cells were lysed by needle homogenization and ultrasonication. Cell lysates were incubated with purified wild-type 3CLpro or inactive mutant 3CLpro C145Q. The proteomes were denatured, and all native N-termini and 3CLpro neo-N-termini were labelled with isotopic formaldehyde. Peptides were digested with trypsin, pooled together, then filtered to isolate peptides with isotopic labels. 3CLpro host cell substrates were identified by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and searching protein databases. Experiment conducted by Pablos *et al*. (11). TAILS method developed by Kleifeld *et al*. (12). Figure created with BioRender.com.

Moreover, 3CLpro has been a candidate therapeutic drug target since the early stages of the COVID-19 pandemic due to its low mutation rate, extensive proteolytic activity, and critical role in SARS-CoV-2 replication (13). However, recent studies have shown that SARS-CoV-2 variants of concern express 3CLpro with missense point mutations (14). These 3CLpro mutants may have altered proteolytic activity or drug inhibition sites (15). Therefore, the suitability of 3CLpro as a therapeutic drug target may need to be re-evaluated.

PROPOSED RESEARCH QUESTIONS

Given the rapid emergence of SARS-CoV-2 variants of concern, it is becoming increasingly urgent to develop and approve therapeutic drugs to reduce the clinical burden. Recently, the 3CLpro inhibitor PAXLOVID^{TM} by Pfizer has been approved for use in Canada and has been shown to effectively treat moderate to severe cases of COVID-19 (16). However, there is a lack of up-to-date research on the vast number of 3CLpro host substrates, as well as the implications of 3CLpro mutations on its status as a desirable drug target. Elucidating the structure and mechanism of 3CLpro at the molecular level is fundamental to understanding why SARS-CoV-2 requires so many host targets, and in turn, will help us approve safe and effective therapeutics. This article will explore the newly-discovered host cell degradome of 3CLpro and discuss its relevance as a therapeutic drug target. First, I will review select host cell proteins that are cleaved by 3CLpro and describe their implications for the antiviral

response. Second, I will explain whether 3CLpro mutations will compromise the efficacy of PAXLOVID™ against future SARS-CoV-2 variants of concern.

PROPOSED PROJECT NARRATIVE

Which host cell proteins are cleaved by 3CLpro, and how does this subvert the host immune response against infection? Using TAILS, 3CLpro was found to proteolyze over 100 host cell proteins, many of which are involved in essential cellular processes (11) (Fig. 3). For example, 3CLpro cleaves polypyrimidine tract-binding protein 1 (PTBP1), a protein that is involved in pre-mRNA alternative splicing in healthy cells (17). PTBP1-induced splicing results in the translation of mRNA for neuronal cell differentiation, T cell activation, embryonic and erythrocytic development, and apoptosis (18). To enable these functions, PTBP1 has a nuclear localization sequence (NLS) that allows it to be shuttled between the nucleus and cytoplasm (18). Interestingly, Pablos *et al*. (11) found that 3CLpro specifically cleaves the NLS of PTBP1, resulting in an altered PTBP1 subcellular localization and a greater proportion of PTBP1 in the cytoplasm of 3CLpro-incubated cells (Fig. 3A). The proximity of 3CLpro to the ER-associated RTC (10) could be hypothesized to allow for proteolysis to occur. This provides evidence that 3CLpro strategically targets substrates to subvert translation and cell differentiation, and ultimately represses antiviral responses such as lymphocyte activation and apoptosis of infected cells.

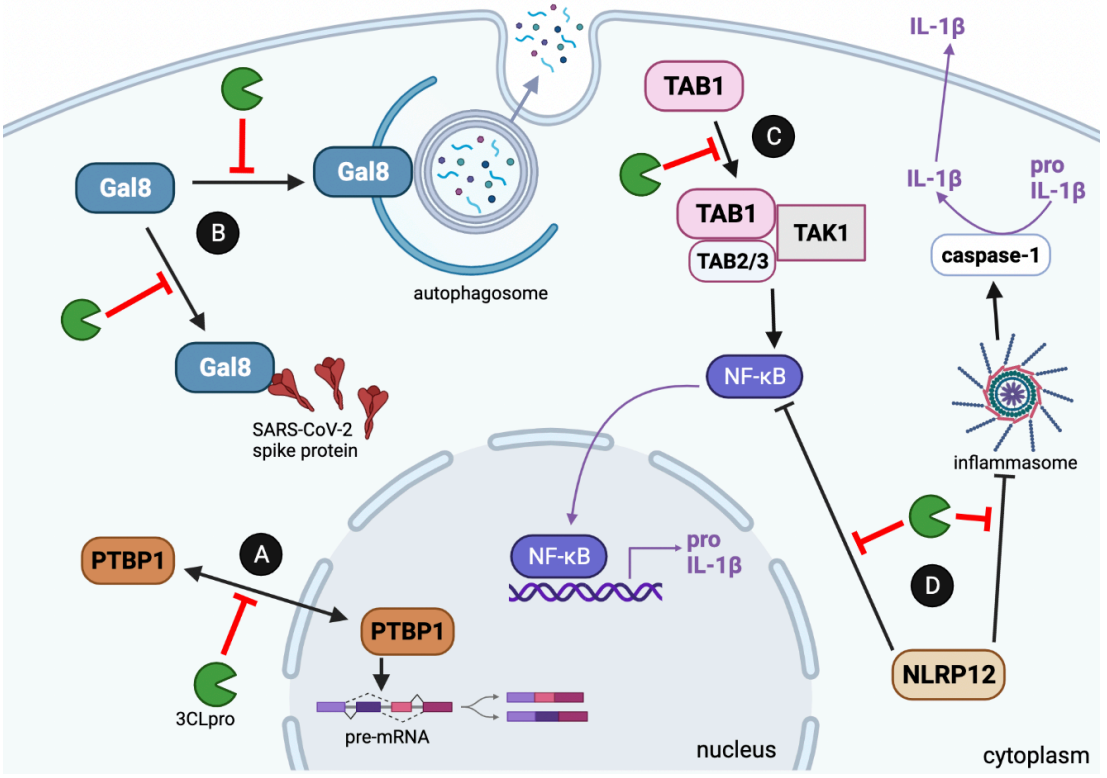

FIG. 3 3CLpro cleaves host cell proteins involved in essential host processes and the antiviral response. (A) 3CLpro cleaves the NLS of PTBP1, preventing PTBP1 from being shuttled between the cytoplasm and the nucleus and regulating alternative mRNA splicing. (B) 3CLpro cleaves galectin-8, inhibiting autophagy and binding to the SARS-CoV-2 spike protein to induce xenophagy. (C) 3CLpro cleaves TAB1, preventing the activation of transcription factor NF-ϰB and production of pro-inflammatory cytokine IL-1β. (D) 3CLpro cleaves NLRP12, resulting in NF-ϰB and inflammasome over-activation, and greater production of mature IL-1β by caspase-1. All processes depicted in the figure are largely simplified. Figure created with BioRender.com.

In addition to cellular translation, 3CLpro cleaves the host protein galectin-8 (Gal8) to inhibit cellular autophagy (11) (Fig. 3B). Gal8 binds glycans and targets damaged or pathogen-containing vesicles for autophagy (19). During SARS-CoV-2 infection, Gal8 binds the heavily-glycosylated spike protein RBD (20) to induce xenophagy, or selective autophagy, as an antiviral mechanism (11). For this reason, galectin inhibitors have been investigated as indirectly-acting antiviral drugs against SARS-CoV-2 (21). However, 3CLpro was found to cleave Gal8 at the glycan-binding site, directly inhibiting Gal8-RBD binding for xenophagy and preventing the host cell from eliminating viral protein- and RNA-containing vesicles (11). Taken together, this new insight on Gal8 proteolysis negates the effectiveness of galectin inhibitor drugs, and rather validates the benefit of directly targeting 3CLpro instead.

Furthermore, 3CLpro cleaves host cell proteins of the innate immune system, particularly those involved in cytokine signalling. Pablos *et al*. (11) found that 3CLpro proteolyzes TGF-beta activated kinase 1 binding protein 1 (TAB1) (Fig. 3C). TAB1/2/3 are responsible for the activation of Mitogen-activated protein kinase kinase kinase 7 (MAP3K7 or TAK1), which in turn upregulates NF-κB expression (22). During SARS-CoV-2 infection, 3CLpro cleaves TAB1 in two cut sites (22), thereby preventing the host from initiating type I interferon signalling. In a different study, Moustaqil *et al*. (22) demonstrated that 3CLpro cleaves nucleotide-binding oligomerization-like receptor protein 12 (NLRP12), a negative regulator of inflammasome formation (Fig. 3D). NLRP12 also negatively regulates pro-inflammatory cytokine production, including IL-1β (23). In contrast to TAB1 cleavage, 3CLpro cleavage of NLRP12 results in uncontrolled formation of the inflammasome and overproduction of NF-κB and IL-1β. These findings could explain the hyperinflammatory response in COVID-19 patients (22), and justify investigating 3CLpro as a drug target to alleviate adverse symptoms of the disease.

Will mutations in 3CLpro compromise the efficacy of 3CLpro inhibitor drugs against future SARS-CoV-2 variants of concern? In January 2022, Pfizer's 3CLpro inhibitor drug PAXLOVID™ was approved in Canada for the treatment of moderate to severe cases of COVID-19 (24). PAXLOVID™ consists of two orally administered pills called Nirmatrelvir and Ritonavir, which inhibit the 3CLpro active site and slow the host's metabolism of Nirmatrelvir, respectively (25). Pfizer reported that COVID-19 patients who received PAXLOVID™ within three days of the onset of symptoms had 89% lower risk of hospital admission or death, compared to a placebo pill (16). PAXLOVID™ presented an ideal treatment option due to its ease of administration in pill-form, and being well-tolerated in humans (16). Furthermore, 3CLpro has high sequence identity with homologs in other coronaviruses, such as 96% similarity with SARS-CoV, 87% with MERS-CoV, 90% with human-CoV and 90% with bovine-CoV, thus reducing the likelihood of 3CLpro mutating to the point of antiviral resistance (6).

However, emerging SARS-CoV-2 variants of concern have been shown to harbor missense mutations in 3CLpro (14). This has led researchers to be skeptical of Pfizer's data showing reduced rates of hospitalization in COVID-19 patients taking PAXLOVID™. For example, the B.1.1.284 variant carries a Pro108Ser mutation in 3CLpro that reduces its enzymatic activity, suggesting that this attenuated 3CLpro mutant had resulted in the milder COVID-19 symptoms, and not PAXLOVID™ by itself (26). Moreover, the missense mutations in 3CLpro call into question Nirmatrelvir's efficacy in inhibiting the 3CLpro active site in future SARS-CoV-2 variants of concern (14).

To address these concerns, a recent study by Ullrich *et al*. (14) compared the 3CLpro missense point mutations in the Delta, Lambda, B1.1.318, B1.2, Beta, Zeta, and Omicron variants. All mutations occurred outside of the catalytic dyad, and thus an overlay of the six protein structures revealed a conserved 3CLpro structure among SARS-CoV-2 variants (14). The authors conducted a Förster resonance electron transfer (FRET) assay with purified 3CLpro mutants and demonstrated that all mutants were enzymatically active. Next, in a 3CLpro inhibition assay using Nirmatrelvir, all 3CLpro mutants had similar % inhibition by Nirmatrelvir. Thus, PAXLOVID™ may potentially remain effective in new variants of concern (14).

However, another study by Baloch *et al*. (15) performed an *in silico* mutational analysis of the spike protein, RNA-dependent RNA polymerase (RdRp) and 3CLpro. They identified 92 mutations in the spike protein, 37 in mutations in RdRp, and 11 mutations in 3CLpro. 3 out of the 11 3CLpro mutations were found to be deleterious and could alter the structure or function of 3CLpro (15). Although 3CLpro has remained conserved in SARS-CoV-2 variants up until now, these findings remind us that mutations could lead to resistance to PAXLOVID™. Taken together, the emergence of SARS-CoV-2 variants of concern remains an evolving area of research, and only time will tell how long 3CLpro inhibitor drugs will remain effective.

POTENTIAL IMPACT/CONCLUSIONS

A complete understanding of the structure, function, and evolution of 3CLpro is critical to assess its suitability as a therapeutic drug target for SARS-CoV-2 variants of concern. Despite the previous knowledge of 3CLpro's role in the SARS-CoV-2 polyprotein processing, the 3CLpro substrate repertoire turns out to be more complex than we had once thought. In addition, 3CLpro has been found to carry mutations in emerging SARS-CoV-2 variants. These new insights have implications for its effectiveness as a drug target, at a molecular level as well as in the long-term against variants of concern.

Recent work by Pablos *et al*. has identified over one hundred host cell proteins that are subject to 3CLpro proteolysis (11, 22). Select host substrates are characterized in this article, and their cleavage disrupts essential host cell processes such as autophagy (11, 19) and cell differentiation (11, 17, 27), as well as antiviral defenses including xenophagy, apoptosis (18) and cytokine signalling (11, 22, 23). Although the analysis in this article hardly scratches the surface of the 3CLpro host cell degradome, it nonetheless provides insight into the under-studied functions of this multifaceted viral protease. This new knowledge further rationalizes the usefulness and versatility of 3CLpro inhibitor drugs for COVID-19 patients. Ultimately, 3CLpro inhibition mitigates two independent viral processes: 1) SARS-CoV-2 replication, and 2) disruption of host antiviral responses to infection (11). By blocking these two processes, 3CLpro inhibitor drugs shut down viral pathogenesis, as well as protect the host. The substrate specificity of 3CLpro is certainly complex, but Pablos *et al*. have laid the groundwork for future studies to characterize more host substrates involved in antiviral defenses against SARS-CoV-2.

Researchers have made considerable progress in the development of antiviral therapeutics. As of February 2022, Health Canada has approved remdesivir (28), bamlanivimab and etesevimab, casirivimab and imdevimab (29), sotrovimab (30), and most recently, PAXLOVID™, for the treatment of COVID-19 (31). Of note, PAXLOVID™ is the first oral therapeutic in Canada that can be administered at home (31), circumventing the need for treatment in a hospital. Pfizer has demonstrated that PAXLOVID™ reduces the risk of hospitalization in COVID-19 patients (16), and recent studies have validated 3CLpro's conserved structure (6, 7) and enzymatic activity (14) among mutants. Although this is promising data to support the use of PAXLOVID™ to treat variants of concern, missense mutations in 3CLpro could lead to altered activity (26). This could potentially result in resistance to the drug if it continues to be administered as a monotherapy (32, 33).

To prevent the emergence of drug-resistant variants, a possible solution would be to administer PAXLOVID™ with a second drug, with a distinct mechanism of action, as a combination therapy (34). For instance, bamlanivimab and etesevimab are neutralizing monoclonal antibody treatments that target different epitopes of the SARS-CoV-2 spike protein (35). A clinical trial showed that the combination of bamlanivimab and etesevimab resulted in significant reduction of viral load in COVID-19 patients compared to bamlanivimab monotherapy, and the authors predict that this multidrug therapy will decrease the frequency of future resistant variants (35). Likewise, PAXLOVID™ could be administered in combination of another approved drug with a different mode of action. For example, if PAXLOVID™ is determined to be compatible with remdesivir, an RdRp inhibitor (28), these two drugs could simultaneously target SARS-CoV-2 polyprotein processing and mRNA replication. After conducting extensive research to determine the feasibility and safety

of a given multidrug therapy, the efficacy PAXLOVID^{TM} would be improved and the risk of deleterious 3CLpro mutants would be reduced.

Ultimately, it is difficult to predict the timeline of 3CLpro mutant emergence and antiviral drug resistance, presenting a major challenge to designing the perfect drug. Nonetheless, the research and knowledge about 3CLpro has incredibly progressed, and will lead to informed decisions regarding 3CLpro inhibitor therapeutics and will reduce the burden of the COVID-19 global pandemic for all.

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