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The development of papain-like protease from SARS-CoV-2, a potential drug target for antiviral screening: A review

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ABSTRACT The SARS-CoV-2 outbreak caused a global pandemic, claiming numerous lives and becoming this century's most widespread life-threatening disease. The virus relies on two specific enzymes to facilitate replication, 3-chymotrypsin-like protease (3CLPro) and papain-like protease (PLpro). These enzymes are crucial in breaking down nonstructural polypeptides into functional proteins. PLpro with LXGG↓X recognition and cleavage sites also play a role in deubiquitylase (DUB) and delSGylase by cleaving after the double glycine residue of ubiquitin (Ub) and ISG15 as a mechanism to suppress the host's innate immune response. Despite its important role in the viral infection cycle and the potential for drug discovery, no antivirals have been approved as PLpro inhibitors. Therefore, this review focuses on PLpro protein, its recombinant product development and purification, and its application as a protein target in drug discovery for COVID-19 screening to develop effective COVID-19 drugs.

KEYWORDS Drug discovery; Papain-like protease; SARS-CoV-2

1. Introduction

In December 2019, a new type of coronavirus called Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was discovered in Wuhan, China (Wang et al. 2020). In the past, certain strains of Coronaviruses have led to worldwide epidemics, including MERS-CoV in 2012 and SARS-CoV in 2002 (Goldsmith et al. 2004; de Groot et al. 2013; Lu et al. 2020). SARS-CoV-2 is a virus with a crown-like shape that causes influenza-like illness, highly pathogenic, and widespread in various countries (Cao 2020; Hartenian et al. 2020; Kakodkar et al. 2020; Naqvi et al. 2020). According to the World Health Organization (WHO), as of February 21, 2023, the confirmed numbered of COVID-19 cases globally had reached around 757 million, with approximately 6.85 million reported deaths attributable to COVID-19 (WHO 2023).

The primary way that SARS-CoV-2 spreads is by respiratory droplets that are released when an infected person talks, coughs, or sneezes. Droplets containing the virus may enter people's noses or mouths, resulting in COVID-19 infection. Additionally, COVID-19 can be spread through contact with surfaces contaminated with the virus, followed by touching one's mouth, nose, or eyes (Harrison et al. 2020; Rando et al. 2021; Sachs et al. 2022).

Several strategies to combat COVID-19 include vaccination to generate herd immunity (Frederiksen et al. 2020; Corti et al. 2021; Rosenberg et al. 2022), antiviral therapy (Beigel et al. 2020; Cully 2022; Hammond et al. 2022), and antibody therapy (Baum et al. 2020; Taylor et al. 2021).

The SARS-CoV-2 genome RNA contains information to produce two proteases, PLpro and 3CLpro (Hartenian et al. 2020). Those proteases enable viral protein maturation through the cleavage of the nonstructural proteins (nsp) (Amin et al. 2021; Kuo et al. 2021; Yu et al. 2022). PLpro, with a catalytic triad of C111, H272, and D286, has three cleavage sites for cleaving and maturing proteins nsp 1, nsp 2, and nsp 3 (Armstrong et al. 2021; Razali et al. 2021) (Figure 1). On the other hand, 3CLpro, with catalytic dyads H41 and C145, cuts more with 11 cleavage sites for cleaving and maturing proteins nsp 4 to nsp 16 (Bera et al. 2021; Razali et al. 2021). Interestingly, unlike 3CLpro, which only plays a role in cleaving and maturing nsp, PLpro with LXGG↓X recognition sequence, where X is any amino acid, affects the host's immune response by regulating the removal of a molecule called ISG15 through deubiquitylation. This mechanism will prevent the activation of signals from pattern recognition receptors for triggering the host's immune response to viruses (Freitas et al. 2020; Shin et al. 2020; Amin et al. 2021; Lim et al. 2021). However, no antiviral has been used clinically with

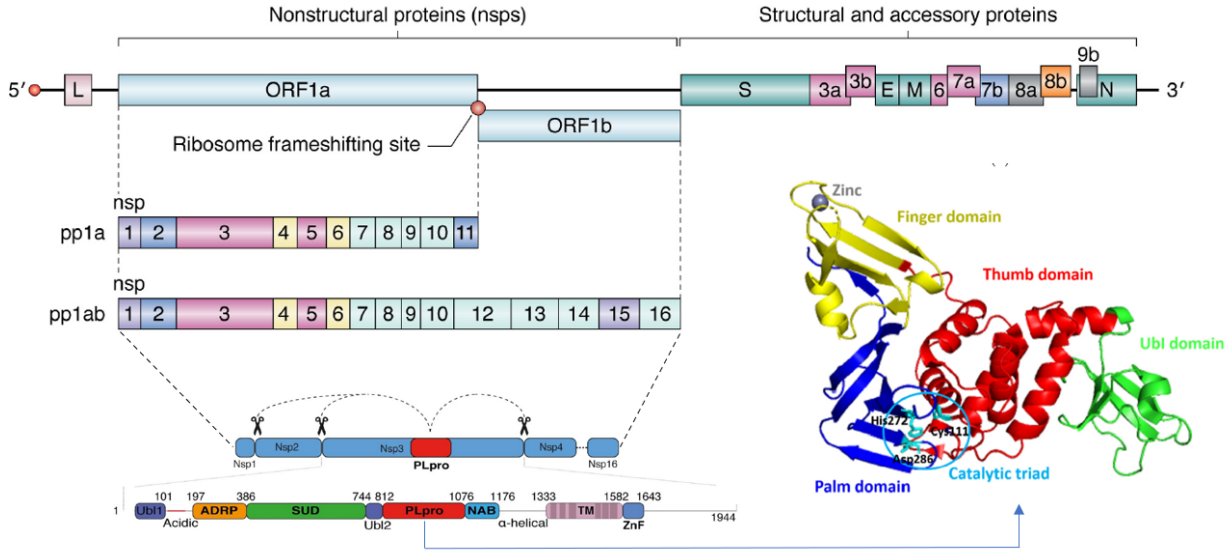


FIGURE 1 The genome RNA structure of the SARS-CoV-2 virus and PLpro structure (Hartenian et al. 2020; Armstrong et al. 2021; Razali et al. 2021).

a mechanism as a PLpro inhibitor. This review discusses the development of PLpro recombinant protein as a target receptor and its applications in antiviral screening.

2. SARS-CoV-2 Structure and Mechanism of Infection

2.1. SARS-CoV-2 structure

The coronavirus group contains viruses that can infect animals and humans, such as SARS-CoV and MERS-CoV. In humans, these viruses are capable to causing respiratory infections that range in severity from mild to severe (Hu et al. 2021). Until now, there have been three outbreaks due to coronavirus, namely SARS, MERS, and a most recently COVID-19 (Dlamini et al. 2020). Lockdowns were imposed in nearly every country in response to the COVID-19 pandemic (Zheng 2020; Onyeaka et al. 2021).

The scientific community classifies SARS-CoV-2 as a member of the *Betacoronavirus* genus, which belongs to the Coronaviridae family and the Coronavirinae subfamily (Hartenian et al. 2020). According to the Baltimore classification, SARS-CoV-2 belongs to group IV, characterized by a single-stranded RNA genome with a relatively extensive size of approximately 30 kb (Ryu 2017; Kim et al. 2020). It is composed of a single-stranded RNA genome and four major structural proteins, i.e., the trimeric spike glycoprotein (S), nucleocapsid (N), membrane protein (M), and small envelope membrane protein (E) (Yao et al. 2020; Rando et al. 2021) (Figure 2). The genomic RNA coding for structural, nonstructural, and accessory proteins plays a critical role in cellular viral regulation, including viral infection and replication of new virion (Kim et al. 2020; Naqvi et al. 2020; Osipiuk et al. 2021; Zhang et al. 2022).

Spike glycoprotein (S) will recognize and attach to the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of human cells. This binding event is the entry point for the virus to penetrate and infect the host (Harrison et al. 2020; Lan et al. 2020; Yao et al. 2020). The N protein protects the viral genome from damage and is packed into a ribonucleoprotein complex during virion assembly (Zeng et al. 2020; Bai et al. 2021). The M protein is a component of the viral envelope, essential in several aspects of viral assembly. In particular, it aids in binding other structural proteins, stabilizes the nucleocapsid, and facilitates the final stages of assembly (Zeng et al. 2020; Bai et al. 2021). Small envelope membrane protein (E) is the smallest structural protein that releases the viral genome, viral assembly, budding, and cell lysis (Harrison et al. 2020; Yao et al. 2020). Due to the presence of lipids and proteins on the surface of SARS-CoV-2, the virus is susceptible to elimination through the application of detergent, whether it is present on the human body or various surfaces (Ryu 2017; Hardison et al. 2022).

The SARS-CoV-2 genome sequence shares a significant amount of similarity with the SARS-CoV genome se-

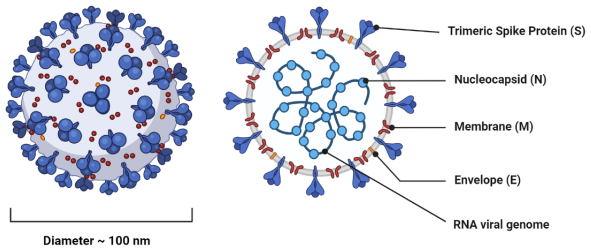


FIGURE 2 The arrangement of the SARS-CoV-2 virus (Yao et al. 2020; Rando et al. 2021).

quence (ranging from 79% to 96%), and with the MERS-CoV genome sequence, with a similarity of 50% (Yang et al. 2020; Hu et al. 2021). As a result, the amino acid composition and structure of protein SARS-CoV-2 are comparable to those of SARS-CoV, with both viruses having an open reading frame (ORF) arranged in the order of 5′-cap to 3′-poly(A) tail, namely ORF1ab, which encodes 16 nsp and four structural proteins (Yang et al. 2020; Hu et al. 2021). Nonetheless, there is little homology between the spike (S) protein, ORF8, ORF3b, and ORF10 genome sequences (Yang et al. 2020). While both SARS-CoV and SARS-CoV-2 recognize and bind to the ACE2 receptor, their S1 subunits differ, resulting in a difference in the location of the receptor binding domain (RBD) (Hu et al. 2020).

2.2. Mechanism of infection

The spike protein found on the surface of the virion is responsible for initiating receptor binding and membrane fusion (Lan et al. 2020). The spike protein is composed of two subunit proteins, S1 and S2. The S1 subunit contains the RBD, which straight interacts with the ACE2 receptor on human cell surfaces. Multiple hydrogen bonds and other interactions facilitate the binding of the RBD to the receptor. Once the binding occurs, the spike protein undergoes a structural change that exposes a site between two subunits (S1 and S2). This site can be cleaved by either the cellular enzyme cathepsin L or the transmembrane protease serine 2 (TMPRSS2). The cleavage is necessary for the virus to enter the host cell and release its genetic material (Harrison et al. 2020; Jackson et al. 2022).

Upon entry into the host cell, the virus will discharge the viral genetic material. The single-strand positive RNA genome has two open reading frames (ORF1a and ORF1b) that will be used directly as a template to synthesize polyproteins by the host cell ribosome. The polyprotein will then be processed by two proteases, PLpro on nsp3 and 3CLpro on nsp5, to produce 16 nonstructural polyproteins essential for viral replication. The RNA-dependent RNA polymerase (RdRp) that originates from the nsp12 is responsible for both copying and transcribing the viral RNA into both the viral RNA genome and a sequence of subgenomic RNAs (Kim et al. 2020; Yao et al. 2020; Amin et al. 2021; Kuo et al. 2021).

The subgenomic RNAs serve as a blueprint for producing various components of the SARS-CoV-2 virus, including the structural and accessory proteins that regulate innate immunity, viral replication, and disease severity. All of these components are created using the host cell's ribosomes during the translation process (Harrison et al. 2020; Hartenian et al. 2020).

New virus particles are formed within the host cell by the assembly of both the newly synthesized structural proteins and the genome RNA. The assembly process begins with the formation of the ribonucleoprotein complex by the nucleocapsid and genome RNA. The other structural proteins form the envelope, including the ribonucleoprotein complex as the core. This process can trap a place in the endoplasmic reticulum (RE) and utilizes lipids from the ER as part of the viral particle membrane. The new virus will be transported to the cell surface and released from the host cell by budding from the cell membrane into the extracellular space, where it can infect other host cells and continue the cycle (Hartenian et al. 2020; Naqvi et al. 2020).

3. Potential Therapy for COVID-19

3.1. Antiviral

Ritonavir/Nirmatrelvir (Paxlovid) is a 3CLpro inhibitor that has obtained Emergency Use Authorizations (EUA). The EUA is a permit for using certain drugs and vaccines in emergencies threatening public health. Regulatory authorities grant EUA based on whether the product has sufficient scientific evidence of safety and efficacy and whether there are no other options or therapies that are sufficient and officially authorized to treat the illness. Ritonavir/Nirmatrelvir is recommended as a first-line treatment for non-severe cases of COVID-19 by NIH guidelines and WHO. Nirmatrelvir acts as a 3CLpro inhibitor, combined with Ritonavir as a boosting agent by inhibiting CYP3A4, and increases the half-life of Nirmatrelvir (Cully 2022; National Institute of Health (NIH) 2022; Tan et al. 2022).

The FDA has approved Remdesivir as the sole antiviral medication that works as an inhibitor of RdRp. This antiviral, administrated by injection, acts as a chain terminator that inhibits RdRp in the elongation process of RNA and stops the virus's replication. Remdesivir is a broad-spectrum antiviral discovered by drug repurposing with the first indication for the Ebola virus. For COVID-19, this drug effectively reduces illness duration (Cully 2022; National Institute of Health (NIH) 2022).

Another RdRp inhibitor, Molnupiravir, has already obtained EUA. Healthcare providers can use Molnupiravir as an alternative regimen for COVID-19. Unlike Remdesivir, Molnupiravir's mechanism did not terminate the elongation process but induced mutagenesis that will lead to virus death (Kabinger et al. 2021; Cully 2022; National Institute of Health (NIH) 2022). However, this drug can potentially be mutagenic and less safe than other COVID-19 drugs. Molnupiravir is also an antiviral discovered by drug repurposing with the first indication for Venezuelan equine encephalitis virus antiviral (Cully 2022).

3.2. Monoclonal antibody and convalescent plasma

Monoclonal antibodies contain four protein chains held together by several disulfide bridges. These chains consist of two heavy chains and also two light chains. Monoclonal antibodies are molecules the immune system produces specifically to identify and destroy foreign molecules (antigens) derived from viruses or bacteria that invade the body. The Fab region on monoclonal antibody has an antigen binding site or a part that will recognize and bind to the epitope of an antigen. Each monoclonal antibody has a different sequence in the Fab region and recognizes a

particular antigen explicitly. The Fc region is part of the heavy chain that can attach to B or T cells. The Fc region has the same sequence in each monoclonal antibody (Sanchez-Trincado et al. 2017).

Developing monoclonal antibodies (mAbs) for COVID-19 begins with searching for sources from the antibodies. The source can be obtained from the blood plasma of a COVID-19 survivor or a humanized mouse that was immunized using antigens from SARS-CoV-2. Furthermore, searching for B Lymphocyte cells that produce specific antibodies against RBD and mAb identification was conducted. In the following process, cloning and expression of mAb were carried out to increase mAb yield, which would then be validated and characterized. The mAb with the best stability and affinity will be selected and formulated as a mAb drug (Taylor et al. 2021).

Regen-COV is an antibody cocktail, or a combination of two monoclonal antibodies (casirivimab and imdevimab) used to treat SARS-CoV-2 infection (Copin et al. 2021). This mAb will bind to the RBD, a particular spike protein segment responsible for recognizing and binding to the ACE2 receptor, thus preventing viral infection. Casirivimab and imdevimab have different binding sites on RBD and chose based on the affinity to the region in RBD that is rarely mutated. This mAb works as a ′neutralizer′ for the virus, so it is included in passive immunotherapy to minimize virulence (Baum et al. 2020; Hansen et al. 2020).

The Omicron variant has exacerbated the COVID-19 pandemic. This new variant is tremendously contagious because it has a greater affinity for binding to ACE2 receptors. Molecular dynamics simulations reveal that the Omicron variant can bind more tightly to ACE2 receptors than the wild type, leading to a higher infection rate. Mutations N440K, T478K, E484A, Q493R, and Q498R in RBD cause an increase in RBD charge, thus increasing RBD's electrostatic interaction with ACE2 (Nguyen et al. 2022). Those mutations caused most of the mAb products for COVID-19, including Regen-COV, to lose their neutralizing ability against the Omicron variant (Fan et al. 2022).

Convalescent plasma is a medical treatment option used to treat COVID-19, which involves administering blood plasma obtained from individuals who have successfully fought off a SARS-CoV-2 infection. Blood plasma is the fluid part of blood comprising antibodies and other proteins. People who have recuperated from COVID-19 have generated antibodies to counteract the SARS-CoV-2 virus. Their blood plasma is rich in these antibodies (Lee et al. 2020). Transferring these antibodies that specifically attack the SARS-CoV-2 virus from recovered patients to those currently infected potentially assists the recipient's immune system in combating the virus. Convalescent plasma therapy can rapidly reduce the viral load and improve clinical outcomes, reducing disease severity and mortality. There are also potential risks associated with convalescent plasma therapy, including transmitting other pathogens. Therefore, the use of convalescent plasma should be carefully considered in a case when other treatment options are not available (Duan et al. 2020).

3.3. Vaccines

The primary strategy to combat COVID-19 is prevention through vaccination to generate herd immunity. A vaccine is a substance designed to trigger the immune system to synthesize antibodies and offers protection against a particular disease. It is typically created using a weakened or inactivated version of the disease-causing agent, which functions as an antigen without causing illness. Multiple COVID-19 vaccine platforms are currently in use, each utilizing a unique approach to generate an immune response. The mRNA platform was developed by Pfizer-Biontech and Moderna using mRNA technology to introduce genetic instructions to human cells, prompting them to produce the spike protein found on the surface of the SARS-CoV-2 virus. This protein is then recognized by the body as an antigen, triggering an immune response and the production of antibodies. This vaccine platform was first approved clinically for COVID-19 (Frederiksen et al. 2020; Corti et al. 2021).

AstraZeneca and Johnson & Johnson have created another approach utilizing viral vectors. The vaccine utilizes a harmless adenovirus as a vector to deliver genetic material that contains the virus's protein to be introduced into human cells. The vaccine employs non-replicating adenovirus or replication-deficient adenovirus as a vector, facilitating the entry of the adenovirus into host cells, stimulating antigen production, and subsequently eliciting an immune response. However, the viral vector lacks the capacity for self-replication, ensuring the adenovirus's inability to replicate (Frederiksen et al. 2020; Corti et al. 2021).

The subunit protein platform developed by Novavax contains the spike protein produced with recombinant DNA technology, purified, and formulated into a vaccine with adjuvant. Inactivated virus platforms such as Sinovac and Synopharm use the SARS-CoV-2 whole virus, activated or deactivated by chemical processes to trigger the human body's immune system (Corti et al. 2021; Jackson et al. 2022). Some vaccines can provide long-term solutions to COVID-19, but virus mutations can reduce vaccine effectiveness.

4. Recombinant PLpro for AntiCOVID-19 Development

The SARS-CoV-2 infection process involves multiple proteins and enzymes. The subunit S1 located in the spike (S), contains RBD at the open state that can recognize the human ACE2 receptor (Ni et al. 2020). Thus, inhibiting ACE2 can avert the virus from entering cells (Ahmad et al. 2021). Another attractive target is TMPRSS2, which also has a role in viral entry by cleaving the spike pro-

tein to bind to ACE2 (Mantzourani et al. 2022; Shirzad et al. 2022). Aside from the anti-entry target, one of the main proteases of coronaviruses, PLpro has a dual role that participates in the viral replication process by processing polypeptide chains and interfering with the host cell's innate immune response (Fu et al. 2021; Zhou et al. 2021).

Many studies have attempted to produce PLpro as an ingredient for antiviral drug discovery. PLpro is a part of nsp3 located in the ORF1a region. The gene encoding PLpro consists of 948 bases, corresponding to 316 amino acid residues with approximately 36 kDa. PLpro is a small part of nsp3 in the open reading frames 1a (ORF1a) region. The PLpro coding gene consists of 948 bp encoding 316 amino acid residues with a theoretical molecular weight of 36 kDa (Arya et al. 2021; Lim et al. 2021). This protein resembles the right-hand fold, which consists of "thumb-palm-fingers", and the structure resembles the ubiquitin-specific protease (USPs). The palm and thumb domains of PLpro have a catalytic triad of C111, H272, and D286 residues as their active site (Klemm et al. 2020; Razali et al. 2021; Shen et al. 2022). Within the PLpro structure, several studies identified a binding site for a ligand that has good potential, specifically the BL2 loop (Shen et al. 2022). This loop is located near the active site, which can change its shape when substrates or inhibitors are present. This loop is adjacent to the active site that can undergo a conformational change in the presence of substrates or inhibitors (Gao et al. 2021; Osipiuk et al. 2021). Several inhibitors have been studied, such as GRL0617 and VIR251 (Gao et al. 2021; Shen et al. 2022).

4.1. Construction of recombinant PLpro

Several studies have attempted to produce PLpro recombinants, shown in Table 1.

The function of PLpro as a protease enzyme makes it a potential agent for COVID-19 drug receptor targets. Several studies have attempted to produce these proteins with recombinant technology and various modifications. The most frequently used expression system is the pET system, especially pET28a, expressed by *E.coli* BL21(DE3) (Shilling et al. 2020). The pET28a system is recombinant with codon optimization and encodes the N-terminal or C-terminal His-tag to assist the purification process with Ni-NTA (Arya et al. 2021; Fu et al. 2021; Gao et al. 2021; Patchett et al. 2021; Xu et al. 2021). In addition, the production process with this construction also inserts the SUMO-tag, leading to a more significant amount of soluble protein (Fu et al. 2021; Gao et al. 2021).

Other systems, such as pET22b, pET11a, pET16b, pET21a, pET21d, pETDuet, and pGEX6P-1, are also used for the expression of PLpro (Rut et al. 2020; Armstrong et al. 2021; Lim et al. 2021; Razali et al. 2021; Zhao et al. 2021; Kulandaisamy et al. 2022; Ulfah et al. 2022). Almost all studies expressed PLpro at low temperatures (16–20 °C) and low isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations with 0.1 mM $ZnSO_4$. One study compared PLpro expression at 37 °C and 18 °C, and the results revealed that only at 18 °C was soluble protein expression found (Razali et al. 2021). Another study stated that expression at 20 °C in the pET21d(+)-rPLpro system produces more insoluble than soluble fractions (Ulfah et al. 2022).

Several researchers have attempted to address the low-yield issue in producing soluble PLpro. One approach is to use fusion proteins that incorporate a highly soluble protein, which can increase the solubility of the protein interest. Recently, Lim et al. (2021) demonstrated that fusing the protein of interest with a solubility-enhancing protein, such as Small Ubiquitin-like Modifier (SUMO), led to a higher yield of soluble PLpro compared to gene constructs without fusion with SUMO. Additionally, other studies have shown that fusion with Glutathione S-transferases (GST) is another effective strategy for increasing the solubility of PLpro (Rut et al. 2020; Armstrong et al. 2021) (Figure 3).

Codon optimization is an advantageous technique for expressing proteins in organisms that do not naturally express these genes (Lipinszki et al. 2018). Since the choices of synonym codons, codon bias can alter between the host and the original organism (Lipinszki et al. 2018). In the process of producing eukaryotic proteins using *E. coli*, there may be a problem that causes errors in protein translation and decreases the levels of tRNA (Choi and Geletu 2018; Lipinszki et al. 2018). Codon optimization can be used, which has been found to increase both protein expression and tRNA abundance (Lozano Terol et al. 2021). In addition, this strategy can minimize protein folding errors that lead to increased protein solubility (Rosano and

TABLE 1 Summary of recombinant PLpro production.

Construct of rPLpro	Plasmid	*E. coli* Strain	References
14-His-SUMO-PLpro	pK27-SUMO	*E. coli* lysY/Iq	(Lim et al. 2021)
6-His-Thrombin site-PLpro	pET28a	BL21(DE3)	(Arya et al. 2021)
GST-PLpro-6-His	pGEX6P-1	BL21(DE3)	(Rut et al. 2020)
6-His-TEV-GST-PLpro	pETDuet	BL21 (DE3)-RIPL	(Armstrong et al. 2021)
PLpro-6-His	pET21d(+)	BL21 (DE3)-RIPL	(Ulfah et al. 2022)
PLpro	pET-11a	Rosetta	(Zhao et al. 2021)
PLpro-6-His	pET21a	BL21 (DE3)-RIPL	(Razali et al. 2021)
6-His-PLpro	pET16b	BL21(DE3)	(Kuo et al. 2021)

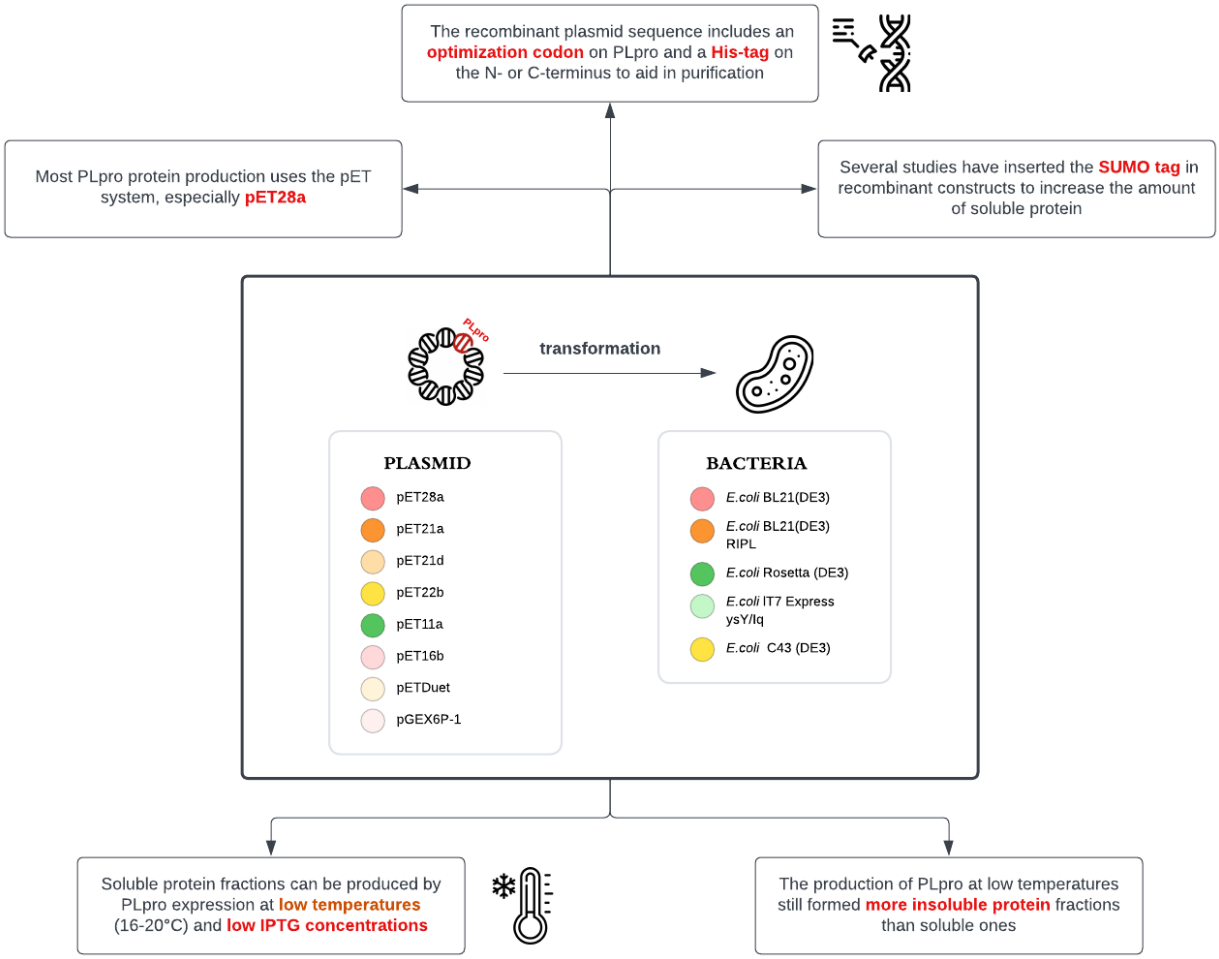


FIGURE 3 Strategy to produce recombinant PLpro which are constructed using certain expression vector and produced from *E. coli* cells system.

Ceccarelli 2014; Lipinszki et al. 2018; Fu et al. 2020).

4.2. Expression of recombinant PLpro

The *E. coli* system is a popular and adaptable method for producing recombinant proteins. Specifically, the *E. coli* BL21 (DE3) strain commonly utilized with T7 RNA polymerase (T7 RNAP) can perform recombinant protein transcription eight times faster than the RNAP strain, which clarifies it as a strong promoter (Wang et al. 2014; Lozano Terol et al. 2021). It also has a deficiency of the protease Lon and OmpT making it suitable to produce heterologous proteins in large quantities to prevent degradation of the heterologous proteins produced. BL21 (DE3)-RIPL and Rosetta are also strains derived from BL21 with the additional ability to increase gene expression with rare codons (Mergulhão et al. 2005; Kayser and Warzecha 2012; Schlegel et al. 2013).

Although the *E. coli* expression system has many advantages for PLpro production, such as growing high-speed and low production costs because *E. coli* can grow to high densities on cheap substrates, most PLpro formed inclusion bodies, protein aggregates with no activity. Specific procedures are necessary to process protein as inclusion bodies, ensuring its eventual refolding into functional soluble protein. Nevertheless, the downstream steps involved in this process are intricate and laborious, thus making the expression of soluble proteins a more favorable option (Kayser and Warzecha 2012; Ulfah et al. 2022).

Furthermore, using chaperone molecules can assist in refolding protein (Khow and Suntrarachun 2012; Rosano and Ceccarelli 2014). Co-expression of this chaperone will prevent the formation of inclusion bodies and increase the solubility of recombinant protein (Khow and Suntrarachun 2012). Not only using chaperone molecules, but the recovery of soluble protein can also increase with low-temperature incubation (Song et al. 2012). Hence the Arctic Express strains, a BL21 strain derivate, apply to these conditions (Francis and Page 2010). These cells express chaperones that can withstand low temperatures (4–12 °C), namely Cpn10 and Cpn60. Two of them can improve the formation of protein structures and increase the yield of soluble and active PLpro (Rosano et al. 2019; Bhatwa et al. 2021).

Besides using the *E. coli* expression system, the bac-

ulovirus expression system, like *S. frugiperda*, can produce large amounts of soluble PLpro. Unlike *E. coli* with limited post-translational modifications (PTM), the baculovirus expression system permits multiple PTMs such as glycosylation, phosphorylation, acylation, and disulfide bond formation (Trowitzsch et al. 2010; Lim et al. 2021). PTM in the baculovirus system can alter the structure of PLpro. PLpro produced by *E. coli* has a higher activity than insect cells (Trowitzsch et al. 2010; Lim et al. 2021).

5. Recombinant PLpro Purification and Downstream Processing

To purify recombinant PLpro, affinity chromatography is typically used, which separates proteins based on their interaction with specific immobilized ligands on a stationary phase. For instance, a ligand such as Ni-NTA or glutathione selectively binds to a protein of interest that contains polyhistidine or GST, respectively. The stationary phase typically comprises resins or gels packed into columns. Upon loading a protein mixture sample onto the column, the immobilized ligand selectively binds to the protein of interest while allowing other proteins to pass through. The subsequent washing of the column eliminates non-specifically bound proteins. The bound proteins are eluted by using specific buffers containing imidazole for Ni-NTA, which disrupts the binding interactions (Janson 2011; Rut et al. 2020; Lim et al. 2021).

Six histidines or 14 histidines are used either at the C-terminal or the N-terminal of PLpro for purification (Rut et al. 2020; Lim et al. 2021). When fusing with polyhistidine or other tagged proteins, adding spacers, such as glycine residues, to prevent steric hindrance that could reduce the protease's ability to cleave the fusion protein (Waugh 2011).

Proteases are used in PLpro purification to cleavage protein tags needed only in expression steps and some parts of the purification process. It should be eliminated because it could alter the PLpro structure. A few studies indicate that polyhistidine tags have a negligible effect; hence they can be disregarded. Some proteases, such as Thrombin (LVPR↓GS), TEV protease (ENLYFQ↓G), and Ulp1 Protease (specifically can recognize and cleave SUMO tags), are commonly used for PLpro and other recombinant proteins (Waugh 2011; Gao et al. 2021; Lim et al. 2021).

During the purification process of recombinant PLpro, various techniques are employed during the polishing process, including ultrafiltration, ion exchange chromatography, and gel filtration. Ultrafiltration involves using a semi-permeable membrane to filter out particles and solutes larger than the membrane's pore size. This process effectively separates small molecules like salts and concentrates the desired proteins (Janson 2011; Lim et al. 2021).

Ion exchange chromatography works based on the principle that protein is charged molecules that can be separated based on their electrostatic interactions with charged resins. Weakly bound proteins can be removed by adding salt concentration into the buffer. Protein has a unique isoelectric point. Changes in pH will affect the charge of the protein and the strength of its interaction with the resin (Jungbauer and Hahn 2009; Janson 2011; Lim et al. 2021).

Gel filtration is used to separate and purify proteins according to their size. This chromatography consists of a stationary phase of polymer resin particles that will filter protein molecules continuously during the elution process with the mobile phase (Janson 2011). Optimizing the flow rate will affect the sample's interaction with the column resin. If the flow rate is low, the sample will diffuse longitudinally and migrate in all directions. Meanwhile, if the flow rate is too high, eddy diffusion occurs, in which molecules migrate through the fluid vortex between the resins, resulting in non-uniform molecule separation (Thenawidjaja et al. 2017).

6. In silico and *In vitro* Studies Designed for PLpro Inhibition Testing

As a protease, short peptide substrates are the leading choice for the design of PLpro inhibition assays. Peptide substrates can be modified by implementing Fluorescence Resonance Energy Transfer (FRET) technology. Anthranilate (2-amino benzoyl/Abz) was attached to the peptide substrate at the N terminal, and nitro-L-tyrosine [Y(3-NO_2)R] at the C terminal and connected. The peptide substrate cleaved by the PLpro will produce a fluorescence intensity from free Abz that can be measured by spectroscopy. (Lim et al. 2021). Substrate design using the peptide sequences RELNGGAYTRYV, FTLKGGAPTKVT, and IALKGGGKIVNNW had cleavage rates of 0.003 ± 0.001 µM/s, 0.09 ± 0.012 µM/s, and 0.01 ± 0.004 µM/s, respectively. The specificity of the FTLKGGAPTKVT substrate for enzymes is proven to produce different cleavage rates with differences of up to nine to 30 times faster than the other (Kuo et al. 2021; Jeong et al. 2022).

Virtual screening of bioactive compounds derived from Indonesian medicinal plants has identified that some plants have the potential to inhibit PLpro, for instance, *Aloe vera*, *Andrographis paniculata*, *Phyllanthus niruri* L., and *Sonchus arvensisi* L. Seco-4-hydroxylintetralin from *Phyllanthus niruri* L. inhibits PLpro most effectively, with a binding energy of –5,68 kcal/mol. The amino acid residues Tyr 273, Gly 163, Thr 301, and Arg 166 form hydrogen bonds with seco-4-hydroxylintetralin, allowing it to bind to PLpro (Firdayani et al. 2022).

Another in silico study on Indonesian medicinal plants reported that curcumin found in *Curcuma longa* Linn. has the potential to inhibit PLpro with a binding energy of –8.45 kcal/mol (Laksmiani et al. 2020). This data aligns with another study that reported that curcumin potentially be a PLpro inhibitor (Rizma et al. 2021).

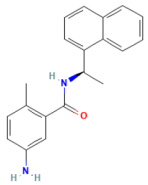
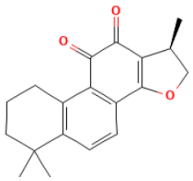
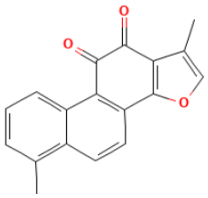
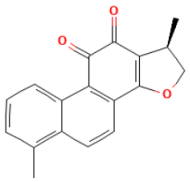
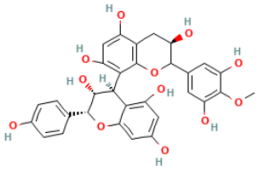
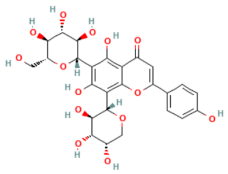
Potential PLpro inhibitors have also been reported from virtual screening of phytochemicals found in Indian medicinal plants. Carvacrol found in black seeds (*Nigella sativa*) has been found to exhibit a favorable binding affinity with the PLpro, as indicated by a binding energy value of –5.8 kcal/mol (Debnath et al. 2022). Another study discovered four of 225 phytocompounds in 28 Indian spices: rutin, luteolin-7-glucoside-40-neohesperidoside, PDT, and DOC, showing potential inhibition activity against SARS-CoV-2 proteases (Rudrapal et al. 2022).

A promising drug candidate for treating COVID-19 was reported in a study with China's 12 most frequently used herbal medicine to treat COVID-19. Liquorice was discovered to possess inhibiting effects against 3CLpro and PLpro among 12, with inhibition rates of 32.85% and 40.93%. Among the 125 compounds from licorice tested, schaftoside has a good affinity with 3Clpro and PLpro, which are –8.4 kcal/mol and –8.5 kcal/mol. Enzymatic assay at eight µmol/L schaftoside has an inhibition rate of 75.9% against 3CLpro and 60% against PLpro. Schaftoside exhibits IC_{50} values of 1.73 ± 0.22 µmol/L and 3.91 ± 0.19 µmol/L against 3CLpro and PLpro, respectively. Additionally, the results indicate that schaftoside is favorable safety and pharmacokinetic characteristics, making it a potential drug candidate for treating COVID-19 (Yi et al. 2022).

Several tanshinone derivatives are potential PLpro inhibitors. "Xuebijing", is a traditional Chinese medicine containing *Salvia miltiorrhiza*, one of the drugs used in China for COVID-19. These drugs reduce damage to multiple organs by inhibiting inflammation and enhancing immune function. Catechins from green tea, cyanovirin-N, several terpene compounds, and in silico propolis derivatives are potential PLpro inhibitors (Jiang et al. 2022).

Repurposing available drugs presents a viable option for discovering potential COVID-19 treatments. This ap-

TABLE 2 PLpro Inhibitors

Compounds	Chemical Structure	IC_{50} (µM)	Reference
GRL-0617		2.21	(Armstrong et al. 2021)
Cryptotanshinone		5.63	(Zhao et al. 2021)
Tanshinone I		2.21	(Zhao et al. 2021)
Dihydrotanshione I		0.59	(Lim et al. 2021)
Proanthocyanidin		2.40	(Kuo et al. 2021)
Schaftoside		3.91	(Yi et al. 2022)

proach offers a rapid and efficient means of identifying antiviral drugs that have already undergone rigorous testing in clinical trials and are deemed safe for human consumption. Protease inhibitor drugs for the Hepatitis C virus (HCV) can inhibit SARS-CoV-2 proteases, such as paritaprevir, grazoprevir, simeprevir, and vaniprevir. Paritaprevir only inhibits PLpro, while others inhibit both proteases. The study also found that HCV drugs that inhibit PLpro work with remdesivir to inhibit virus replication and potentially increase antiviral activity by up to tenfold (Bafna et al. 2021). Mefloquine, an antiviral effective against Human Coronavirus 229E (HCoV-229E), was reported to be also effective against SARS-CoV-2 PLpro. The result of *in vitro* assay showed that Mefloquine has an IC_{50} value of 459 ± 1µM (Kulandaisamy et al. 2022). Further studies and investigations are needed to develop these repurposed drugs as COVID-19 treatment.

7. Prediction of PLpro Inhibitor as an Antiviral with CADD

Computer-aided drug design (CADD) is a method for discovering and developing new drugs using computational tools and techniques (Coumar 2021). The emergence of vast databases containing genomic, chemical, and pharmacological information presents novel drug discovery and repurposing opportunities. In the preliminary phases of drug development, computational prediction is a wise option to procure valuable initial data while economizing costs and time relative to conventional experimental methodologies (Luo et al. 2017). Target-based virtual ligand screening is an effective initial method for searching for drug candidates. This method predicted the potential use of Remdesivir as an RdRp inhibitor in less than two months since SARS-CoV-2 was first identified, using a drug database and a self-built database of natural products (Wu et al. 2020).

Erlina et al. (2022) reported that through virtual screening, several compounds such as 8-methylthio-octyl glucosinolate, sinigrin, and glucoputranjivin contained in Indonesian herbal plants have the potential to become antiviral effects through the PLpro inhibition mechanism. The validation of these results can be confirmed further through experimental tests with enzymatic assays.

8. PLpro Inhibitor Activity Properties for AntiCOVID-19

Several studies have shown that small molecules have the potential as antivirals that inhibit PLpro. Compound GRL-0617 is proven to inhibit PLpro with IC_{50} of 2.21 µM (Armstrong et al. 2021). Zhao et al. (2021) found that cryptotanshinone and tanshinone I, active components derived from *Salvia miltiorrhiza* and commonly used in traditional Chinese medicine (TCM), could inhibit PLpro with IC_{50} values of 5.63 µM and 2.21 µM, respectively. Dihydrotanshione I from *Salvia miltiorrhiza* is also very potent, with an IC_{50} of 0.59 µM (Park et al. 2012; Lim et al. 2021). Potential PLpro inhibitor, shown in Table 2.

In silico and *in vitro* studies have proven that GRL-0617 and its analogs have the potential as antivirals for COVID-19. GRL-0617 works as a PLpro inhibitor by

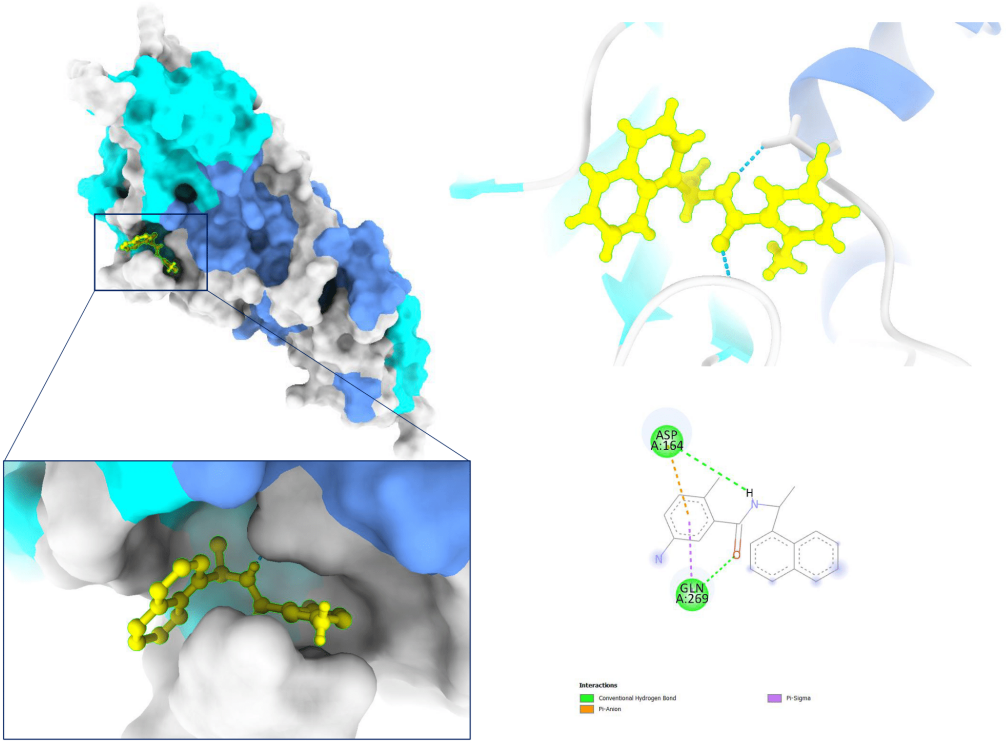


FIGURE 4 Interaction of PLpro with GRL0617.

forming hydrogen bonds with two amino acids, Asp-164 and Gln-269, as evidenced by the PLpro molecule's interaction, corresponding to the PDB ID 7CMD (Figure 4). However, this compound is metabolically labile because it contains the naphthalene ring. So, it is difficult to proceed to the next steps in developing new drugs (Gao et al. 2021; Tan et al. 2022).

9. Conclusions

COVID-19 has emerged as the most significant life-threatening disease caused by the highly pathogenic SARS-CoV-2. The virus constantly evolves and has developed mechanisms to mutate and evade the human immune system, potentially losing efficacy in some treatments (Fan et al. 2022). Extensively used antivirals for COVID-19 treatment may also lead to mutations and inevitable resistance to the current antivirals. RdRp inhibitors showed weaker efficacy than protease inhibitors.

Paxlovid, as a 3CLpro inhibitor, is the first-line antiviral for SARS-CoV-2 infection therapy (National Institute of Health (NIH) 2022). This fact proves that proteases have the potential to be effective target receptors for antiviral discovery. PLpro plays a more critical role than 3CLpro in the virus life cycle because not just for protein cleavage and maturation, PLpro also inhibits the body's immune response. So, screening for PLpro inhibitors will lead to the discovery of novel antivirals with good efficacy. Drug discovery from herbal compounds with PLpro as the target has not been explored, and the potential for obtaining new antivirals is still wide open.

Although it has been widely studied, the expression of PLpro in *E. coli* is quite challenging. Combining particular tag proteins makes it possible to address the issues in inclusion bodies formation and low production of soluble protein to obtain a higher yield of soluble PLpro to be used as a good target for drug discovery.

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Authors' contributions

RN, IH, AM designed the study. RN, MM, FAF collected the literature related to the study. RN, IH, AM analyzed the data. RN, IH, MM, FAF, AM wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The author reports that there are no competing interests.

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