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Article Review

SARS-CoV-2 Proteases: Role and Potential as Drug Target

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Chrecentia Hanna Swestikaputri[1], Tjahjani Mirawati Sudiro[1,2,3*]

[1]Master Programme in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[2]Department of Microbiology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[3]Infectious Disease and Immunology Cluster, Indonesian Medical Education and Research Institute, Jakarta, Indonesia.

Abstract. The coronavirus disease of 2019 (COVID-19) has become a long global pandemic caused by a transmitted and pathogenic virus called Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Even though WHO has retracted the global emergency status of COVID-19, it remains a threat. Various antiviral treatments are being devised and developed due to the coronavirus's high rate of mutation and the need to create more effective treatments for infections. Protease is an important part of the life cycle of SARS CoV-2 hence it is intended as an antiviral target. Several protease inhibitor candidates have been identified, but there is still much to learn, including the structure and mechanism by which these inhibitors inhibit protease. This article investigates the function of proteases in the SARS CoV-2 life cycle and the mechanism of protease inhibition. Past and present research on the protease inhibitor mechanism of action was evaluated in order to generate this literature review. Here we found that the main protease (M^{pro}), one of SARS-CoV's proteases, is highly conserved among coronaviruses and has no human homolog. As a result, numerous M^{pro} inhibitors have been developed in an effort to treat COVID-19. PAXLOVID, an M^{pro} inhibitor, is already approved by FDA for emergency use.

Corresponding Author :
Tjahjani Mirawati Sudiro
Department of Microbiology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
Email : tjahjani.mirawati@ui.ac.id

1. Introduction

In numerous nations, including Indonesia, the coronavirus disease 2019 pandemic, also known as COVID-19, is still active. From January until May 2023, 161,638 fatalities were attributed to COVID-19[1]. Further research resulted in the identification of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) as the cause of the outbreak. In the most severe cases, SARS-CoV-2 is highly contagious and can induce life-threatening viral pneumonia [2]. Despite the fact that vaccinations have progressed and offer a valuable barrier against the virus, a significant number of individuals are either unable to be vaccinated due to pre-existing medical conditions or are refusing to be vaccinated. Moreover, coronavirus has a high mutation rate. Therefore, therapeutics that target SARS-CoV-2 structural and functional proteins are developed [3-4].

SARS CoV-2 is a member of the Betacoronavirus family, which consists of enveloped viruses with single positive-strand RNA (ssRNA+). Once SARS CoV-2 has effectively invaded the host cell, the viral genome will be translated into two polyproteins, pp1a and pp1b. With the aid of viral proteases, both polyproteins will be cleaved into 16 smaller, functional nonstructural proteins. SARS-CoV is known to possess two proteases, specifically papain-like cysteine proteinase (PL2Pro) and 3C-like cysteine proteinase (3CLPro) or M^{pro}. Without cleavage of polyproteins, the process of virus replication cannot continue, and no new virions can be produced [5–8].

To date, the structure and function of important SARS CoV-2 virus proteins, including proteases, have been investigated. Cryo-Electron Microscope (cryo-EM) or X-ray technique is used to examine the structure of the SARS-CoV-2 virus's primary proteins. Not only is the structure of the protein studied but also its biological functions. This can expedite the development of virus-specific therapeutics [6],[9].

M^{pro} is regarded as an ideal target for drug development. There is no homolog of M^{pro} protease in the human body. Moreover, proteases' role in the early stages of SARS CoV-2 replication renders them ideal antiviral therapy targets. It has been studied that M^{pro} inhibitors can inhibit the viral life cycle without causing significant biotoxicity. Besides, its structure has been characterized by a number of studies [10-11]. Lastly, M^{pro} is conserved among coronavirus. The amino acid sequence of SARS-CoV-2 M^{pro} is 96% identical to that of SARS-CoV.[12]

The continuation of research on the structure and function of M^{pro} and the hunt for inhibitors is anticipated to provide an alternative treatment for COVID-19. Nirmaltrevir is a SARS-CoV-2 Mpro inhibitor that exhibits potent in vitro inhibition from all over known human infecting coronavirus. It is one of the antiviral components of the approved drug Paxlovid [13–15].

Prior to this point, there have been numerous studies on the screening of potential compounds targeting M^{pro}, but few on the action mechanism of these compounds. Understanding the mechanism of action can facilitate the discovery of novel compounds and the modification of existing compounds as M^{pro} inhibitors. In this paper, we will examine the function of SARS-CoV-2 protease and the mechanism of protease inhibition, particularly M^{pro}, as an alternative therapy for treating SARS-CoV-2 infections.

2. Experimental Section

2.1. Search Strategy

We conducted a comprehensive and systematic search of multiple databases and other sources of published material to identify relevant studies. Up until May 22, 2023, PubMed, Google Scholar, Google, Scopus were systematically searched for relevant evidence. In addition, the reference lists of completed studies and reviews were combed for additional information. (1) SARS-CoV-2, (2) protease, (3) protease inhibitor, (4) M^{pro} +PLpro, and (5) mechanism of action + protease inhibitor were among the search terms.

2.2. Study Selection

Initial screening is conducted on the basis of keywords and titles followed by removing duplicate articles and guidelines. There were no restrictions on publication or study year, but a greater focus was placed on recent research and updated data. We analyzed studies to learn more about the most recent findings regarding the role of SARS-CoV-2 protease and protease inhibitors as COVID-19 therapy. For the guidelines, we choose the most recently published ones. After that an evaluation was conducted of based on the abstract of the articles. Besides, we performed a screening of online guidelines through their official website. Only eligible guidelines were chosen.

Based on keywords search across all available databases yielded articles and guideline 418 and 112 total results, respectively. We prefer research articles to review articles. From the abstracts to the full-text publications, there were articles with screening potential. Meanwhile, the guidelines were chosen based on the publication date and the coherency of this article outline. 273 of the 368 prospective articles and guidelines were disqualified after screening and assessing. As a consequence, 60 records satisfied the inclusion criteria and were ready for review. Figure 1 illustrates the study selection process.

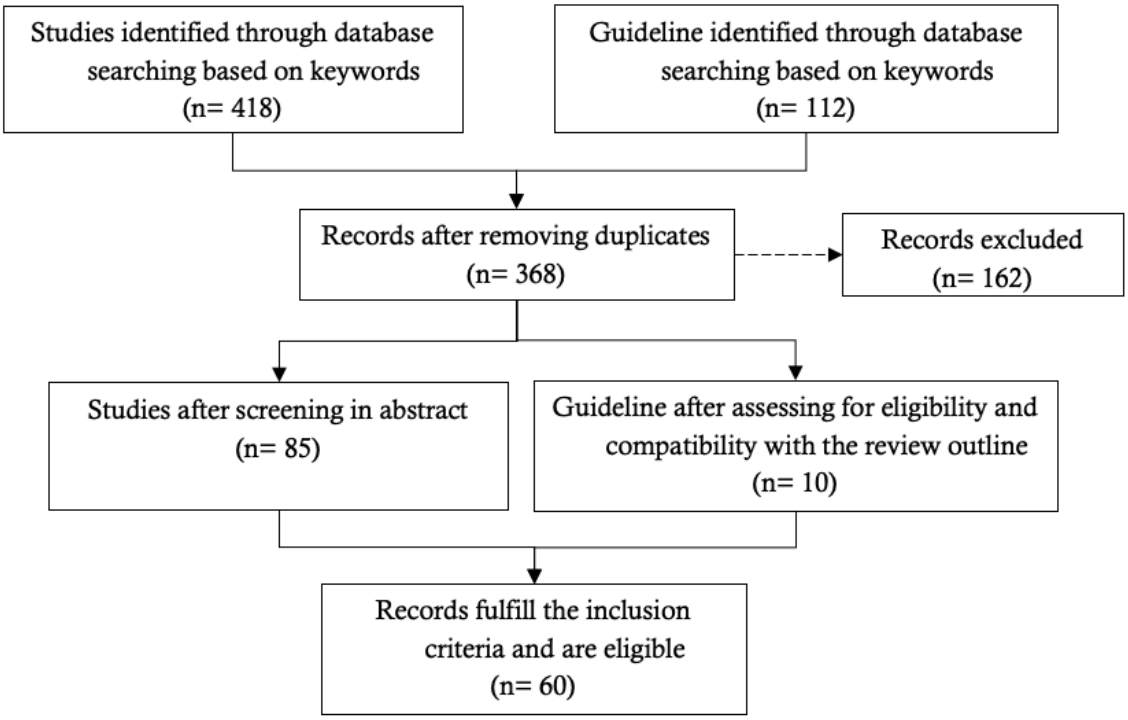

Figure 1. PRISMA workflow of research

3. Results and Discussion

3.1. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

Coronavirus 2 (SARS-CoV-2) is a virus known as Corona Virus Disease 2019 (COVID-19) that causes acute respiratory illness. Since 2019, this virus has been responsible for a global pandemic. This virus has positive single-stranded RNA and is classified as an enveloped virus. The genome of SARS-CoV-2 is ~29.9 kilobase pair in size [16]. This virus belongs to the genus Betacoronavirus. A coronavirus with a size of 20-80 nm [17]. SAR-CoV-2 is composed of multiple structural proteins, including

nucleocapsid (N), membrane (M), envelope (E), and spike (S) proteins. M, E, and S proteins interact with the lipid bilayer of the virus. Meanwhile, N protein is associated with RNA [18].

Infection with the SARS CoV-2 virus begins with the formation of a bond between antigens and receptors on human host cells. Spike protein (glycoprotein S) is present in the transmembrane of SARS CoV-2. This protein acts as the receptor binding domain (RBD). The RBD binds to the ACE2 receptor on the surface of the host cell [19]. Protein S is composed of an N-terminal signal peptide and two subunits, S1 and S2. The N-terminal S1 subunit of the spike protein binds to the receptor, whereas the C-terminal S2 subunit mediates the fusion of the virus with the host cell membrane [20–24]. Protein S binding to the ACE2 receptor induces conformational changes in the subunit S1, thereby exposing the S2 subunit's cleavage site. The protease responsible for S2 subunit cleavage is dependent on the viral internalization pathway [25].

Based on the location of the virus uncoating process, there are two distinct pathways of SARS CoV-2 internalization processes into cells, namely on the cell surface and in the cytoplasm. The expression level of TMPRSS2 on the target cell surface distinguishes between these pathways. TMPRSS2 is a transmembrane protease serine-type II that is highly abundant in the respiratory, intestinal, and urogenital epithelium [25,26].

If the target cell expresses enough TMPRSS2, uncoating occurred at the target cell surface. In this pathway, TMPRSS2 cleaves the S2' site [27]. In insufficient TMPRSS2, the virus penetrates the cell via endocytosis with the assistance of clathrin protein.[28] In this pathway, the S2 subunit is cleaved within the endosome by cathepsin. S2' site cleavage leads to membrane fusion that creates fusion pore, allowing the release of viral RNA to the cytoplasm. Next, the replication and expression of genes commence [25].

The translational process of the virus is wholly dependent on the translational system of the host cell. ORF1a and ORF1b are the two open reading frames of the RNA genome. From the gRNA, two polyproteins, pp1a and pp1b, will be produced. In addition, proteases are involved in the process of degrading polyproteins into sixteen smaller, functional nonstructural proteins (NSPs). The NSPs formed will play a significant role in replication and transcription. Due to its important function in the replication process, protease is a treatment target for SARS CoV-2 infection [20][29].

NSPs will form a replication-transcription complex (RTC). Each NSP plays a function in the process of replication and transcription. NSP number 1 is not directly involved in the replication process, but it does suppress the immune system of the host cell [30]. As primases, replication cofactors, and endoplasmic reticulum modifiers, NSPs 2-11 facilitate the replication process. The NSP 12 is an RNA-dependent RNA polymerase. In addition to their enzymatic functions, NSPs 13–16 serve as helicases, mRNA-modifying enzymes, and proofreaders [20][29].

RTC will synthesize both genomic RNA and subgenomic messenger RNA (sgmRNA). This synthesis occurs within a double membrane vesicle. Then, gRNA will be translated into nonstructural proteins. gRNA is also capable of serving as a template for RNA synthesis and packaging of new viruses. SgmRNA encodes the structural proteins spike (S) protein, E (envelope) protein, M (membrane) protein, and N (nucleocapsid) protein via ORF2-9b [29].

The translation of sgmRNA occurs partially in the cytosol and partially in the endoplasmic reticulum (RE). Ribosomes present in the cytoplasm facilitate the expression of N proteins. In the RE, proteins S, E, and M will be expressed with the assistance of ribosomes. The structural proteins are then transported to the intermediate endoplasmic reticulum-Golgi compartment (ERGIC). Within the ERGIC, the gRNA encapsulation procedure occurs. By budding, the nucleocapsid and other structural proteins are subsequently secreted [20].

The assembly stage begins with the M and E proteins, which form the envelope of the virus. The viral envelope will then envelop the newly formed nucleocapsid. The S protein will combine to produce a virion that is prepared for exocytosis and subsequent release from the cell [31].

3.2. Definition and Classification of Protease

Proteases, also known as peptidases or proteinases, are enzymes that break down proteins by hydrolyzing their peptide bonds. Therefore, proteases are classified as hydrolase enzymes. The active site of proteases is composed of a binding site and a catalysis site [32].

Proteases are prevalent in organisms because they participate in numerous biological processes, such as the formation of new proteins, DNA replication and transcription, and wound repair. Approximately 2% to 4% of an organism's genes are known to encode for proteases. Proteases catalyse irreversible hydrolysis reactions, so their activity must be strictly controlled. Multiple mechanisms exist within the organism to regulate this activity. The inhibition of proteases with macromolecular inhibitors is one of them [33].

Proteases fragment long-chain proteins (polyproteins) by cleaving the peptide bonds between the amino acids that compose the protein. In humans, 6% of proteins are proteases, whereas in bacteria, viruses, and other organisms, 1-5% of proteins are proteases [32]. Some proteases act on specific peptide bonds, depending on the amino acid residues present on the polypeptide. For example, trypsin breaks peptide bonds at lysine and arginine. However, some other types of proteases can break the entire polypeptide chain into amino acids [32][34].

Proteases possess an intricate structure. This can occur as a result of post-transcriptional modifications to the mRNA encoding the protease, such as alternative splicing and polyadenylation variants. Post-translational modifications include glycosylation and phosphorylation. These modifications are advantageous for signaling activities, such as ligand binding. The involvement of other proteases in protease activity results in a cascade of actions.

Endopeptidase and exopeptidase were initially used to classify proteases according to the position of the target peptide bond. Endopeptidases function by hydrolyzing the peptide bond within the polypeptide to generate multiple polypeptide chains, such as papain and trypsin. Exopeptidases, such as aminopeptidase and carboxypeptidase, hydrolyze the peptide bond at the NH2- or COOH- end to produce amino acids and polypeptide residues [34][35]. However, as more protease varieties were discovered, the basis for division expanded. Based on the MEROPS database, there are 1,206 types of proteases divided into 281 families. Classification of MEROPS families based on similarity of amino acid sequences [35][36].

Proteases can also be categorized according to the amino acid components found on their active site, namely glutamate proteases, aspartate proteases, metalloproteases, serine proteases, cysteine proteases, threonine proteases, and aspargic proteases. In hydrolyzing peptide bonds, glutamate, aspartate, and metalloprotease proteases utilize water molecules as nucleophiles. While serine, threonine, and cysteine proteases use the amino acid series as nucleophiles on their catalytic site, also known as the catalytic triumvirate. This mechanism consists of two distinct phases. In the initial step, the catalytic triad attaches to the substrate, producing an acyl-enzyme intermediate and the initial by-product. In the second stage, water will break the covalent bond between the catalytic triad and substrate to generate the second half-product [34][37]

3.3. Protease's Function in Viruses

Protease is an enzyme that catalyses the separation of polyproteins into multiple proteins without affecting the protein's function. According to their function, there are two categories of proteins in viruses: structural proteins and non-structural proteins. Capsid-forming proteins are examples of structural proteins. Enzymes are examples of nonstructural proteins. Proteases can cleave structural and nonstructural polyproteins or separate structural and nonstructural proteins. Herpes simplex virus type 1 protease contributes to the formation of inner capsid-forming proteins. Hepatitis-C Virus NS2/3 protease performs a role in severing the link between NS2 and NS3 proteins. Picornavirus protease is an example of a protease that breaks the bonds between structural and nonstructural proteins.

Picornavirus contains proteases that function in the cleavage of host cell proteins that function in the translation of host mRNA [38].

3.4. SARS-CoV-2 Protease

During replication, SARS CoV-2 synthesises two polyproteins, pp1a and pp1b. In addition, two proteases will divide both polyproteins into 16 non-structural proteins. The first protease is termed main protease (M^{pro}) or 3-Chymotrypsin-like protease (3CLPRO) due to its similarity to the 3C protease found in picornavirus. The second protease is PLpro, or papain-like protease. Both proteases fall under the category of cysteine proteases. Three cleavage sites on PLpro generate non-structural proteins 1 to 3. M^{pro} possesses 11 cutting sites that generate non-structural proteins 4 through 16 [8][39][40].

M^{pro} is a homodimer of cysteine protease with three domains per protomer. M^{pro} contains a catalytic dyad of cysteine 145 and histidine 41. The binding site is located between the I and II domains. M^{pro} in SARS-CoV-2 has an amino acid sequence that is 96% identical to M^{pro} in SARS-CoV [11][39][40].

Papain-like protease consists of two domains: an N-terminal ubiquitin-like (UbI) domain and a thumb-palm-finger-shaped catalytic domain. The catalytic triad of PLpro consists of Cysteine 111, Histidine 272, and Aspartic acid 286. Eighty-three percent of SARS-CoV-2 PLpro's amino acid sequence is identical to SARS-CoV PLpro [39][40].

3.5. Definition and Classification of Protease Inhibitor

Viral protease inhibitors are protein or non-protein compounds that inhibit the action of the protease in the virus, thereby preventing viral replication [41]. The type of protease that is inhibited can be used to classify protease inhibitors. On the basis of this classification, protease inhibitors are divided into six groups:

1. Cysteine protease inhibitor
2. Aspartyl Protease inhibitor
3. Metalloprotease inhibitor
4. Serin protease inhibitor
5. Glutamate protease inhibitor
6. Threonine protease inhibitor

The binding between a protease inhibitor and its target can be reversible or irreversible. In general, reversible inhibitors form a non-covalent bond with proteases. In contrast, non-reversible inhibitors modify the active site of the protease by forming covalent bonds [41].

Reversible protease inhibitors are divided into competitive, uncompetitive and non-competitive inhibitors. Competitive inhibitors prevent substrate binding by binding to the active site of the protease, competing with substrate for active site residue access. Uncompetitive inhibitors inhibit proteases that have already bound to their substrate. Noncompetitive inhibitors attach to the protease with the same affinity despite the existence of a substrate. Allosteric inhibition is the mechanism utilized by noncompetitive inhibitors [41].

Protease inhibitors that are irreversible are also known as inactivators. The interaction between this type of inhibitor and the protease can alter the active site of the protease, preventing it from binding to the substrate. This type of inhibitor does not always establish a covalent bond with the protease, but certain molecules interact with the enzyme with high affinity [41-42].

3.6. SARS-CoV-2 Antiviral Protease Inhibitor

Numerous molecules that block the replication of the SARS-CoV2 virus have been studied to date. One method is to inhibit the activity of the virus proteases. SARS-CoV-2 encodes two cysteine

proteases, PLpro and 3CLpro (M^{pro}). These proteases are essential for the survival of SARS-CoV-2. Therefore, both are promising targets for antiviral development against SARS-CoV-2 [43–45].

Antiviral protease inhibitors are effective at preventing viral replication and are relatively harmless for human use. Both M^{pro} and PLpro of SARS-CoV-2 are essential enzymes involved in viral replication. By inhibiting this protease, viral replication can be effectively disrupted and the viral burden decreased. Besides, due to the distinct substrate specificity of mammalian protease enzymes, antiviral protease inhibitors might be relatively safe for human use [46-47].

Numerous molecules are being investigated as anti-SARS-CoV-2 proteases. Protease inhibitors are typically administered orally, such as nirmatrelvir [48], lopinavir-ritonavir [49], nelfinavir [50] and darunavir [51]. These medications except nirmatrelvir, are designed to be selective for the viral protease enzyme and have been used in the past to treat HIV and other viral infections.[52] Nirmatrelvir is developed particularly to inhibit SARS-CoV-2. By inhibiting the protease enzyme, these medications can inhibit viral replication, viral burden, and potentially infection severity [47].

M^{pro} inhibitors have been researched and developed more extensively than PLpro inhibitors. M^{pro} is well-preserve within coronaviruses, and its substrate-binding site shares a number of characteristics [53]. In addition, there is no human homolog of M^{pro}. M^{pro}'s substrate recognition sequence (Leu-Gln-↓-Ser-Ala-Gly) is not associated with any human protease known to science. Therefore, M^{pro} inhibitors must have minimal side effects due to their extreme specificity for SARS-CoV-2 [54]. Moreover, M^{pro} is conserved among coronaviruses, unlike spike proteins that show high mutation which are subject to intensive mutagenesis [55].

Despite the fact that a number of peptidomimetic covalent M^{pro} inhibitors have been reported, few candidates are headed to clinical trials [53][54][56]. However, the FDA has authorized the emergency use of Paxlovid (nirmaltrevir + ritonavir) developed by Pfizer, for the treatment of mild to moderate symptoms in adults and children COVID-19 [57]. However, based on the clinical trial phase2-3, paxlovid can reduce risk by 89% compared to placebo for severe COVID-19. In addition, no safety concerns are apparent [58]. According to the Pedoman Tatalaksana COVID-19 edisi 4, Paxlovid is an anti-SARS-CoV-2 drug that can be used to treat severe symptoms [59].

3.7. Mechanisms of Action for Protease Inhibitors

Protease inhibitors prevent SARS-CoV-2 protease from functioning normally by binding to its active site. Protease inhibitors inhibit the protease's ability to cleave viral polyproteins, which are required for viral replication, by adhering to the active site. This inhibits the production of the viral proteins necessary for the assembly of new virus particles [60].

Following binding to the active site of the protease enzyme, the protease inhibitor forms a stable complex that prevents the enzyme from cleaving its target substrates. As a result, the process of viral replication is disrupted, and the production of new infectious viral particles is reduced [60].

The protease M^{pro} is a target for inhibition because it plays an important role in the viral replication process. N3, a Michael acceptor, is one of the molecules that has been shown to inhibit M^{pro}. Michael acceptors are a class of carbonyl-conjugated compounds that have been shown to non-reversibly inhibit the action of cysteine proteases [39]. This molecule creates a covalent bond with the cysteine on the protease's catalytic site. Multiple hydrogen bonds are formed between N3 and the substrate side, securing the inhibitor within the pocket-shaped substrate binding area. The N3 molecule attached to the active site inhibits the binding of the natural substrate M^{pro}, preventing proteolysis. Therefore, SARS-CoV 2 replication is inhibited [6].

Paxlovid, consist of nirmatrelvir and ritonavir, is an antiviral medication created by Pfizer for the treatment of COVID-19. It is designed to specifically target the SARS-CoV-2 protease M^{pro}, also known as 3CLpro. Nirmatrelvir's mode of action involves inhibiting the activity of this viral protease [48]. Ritonavir is not a protease inhibitor but is included in the treatment to increase the concentration and duration of nirmatrelvir by inhibiting its metabolism [57].

Nirmatrelvir was developed for mimicking a peptide that obstructs M^{pro}. It is a covalent inhibitor, which means it establishes a strong, reversible bond with the catalytic cysteine residue (Cys145). By binding to the active site, nirmatrelvir prevents the protease from cleaving polyprotein precursors which form the RTC necessary. This inhibition prohibits the formation of new virus particles [60].

The highly stable covalent bond between nirmatrelvir and the protease enzyme permits protracted inhibition of the enzyme's activity. This sustained inhibition inhibits the protease and reduces viral replication in infected cells. Nirmatrelvir's unique molecular structure is optimized for binding to the active site of the viral protease. This binding increases the drug's effectiveness against SARS-CoV-2 while minimizing its effect on other cellular processes [60].

4. Conclusion

WHO has announced that COVID-19 no longer a global health emergency. In addition, the vaccinations have progressed. However, COVID-19 remains a treat. Thus, finding and further development of new therapeutics for novel coronaviruses (CoV) is crucial. This review is focused on the role of protease and the mechanism of protease inhibitor action to combat SARS-CoV-2. It will aid researchers in the discovery and development of new effective antiviral compounds. The N3 molecule is one of the molecules that have been evaluated to inhibit irreversibly the activity of M^{pro}, but it has not yet reached the clinical trial phase. Otherwise, Paxlovid as an oral antiviral agent already got FDA approval and displayed a promising treatment for patients with COVID-19. Yet, additional testing is required to monitor both the safety and efficacy of Paxlovid in the treatment of COVID-19.

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