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Main and papain-like proteases as prospective targets for pharmacological treatment of coronavirus SARS-CoV-2

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The pandemic caused by the coronavirus SARS-CoV-2 led to a global crisis in the world healthcare system. Despite some progress in the creation of antiviral vaccines and mass vaccination of the population, the number of patients continues to grow because of the spread of new SARS-CoV-2 mutations. There is an urgent need for direct-acting drugs capable of suppressing or stopping the main mechanisms of reproduction of the coronavirus SARS-CoV-2. Several studies have shown that the successful replication of the virus in the cell requires proteolytic cleavage of the protein structures of the virus. Two proteases are crucial in replicating SARS-CoV-2 and other coronaviruses: the main protease (Mpro) and the papain-like protease (PLpro). In this review, we summarize the essential viral proteins of SARS-CoV-2 required for its viral life cycle as targets for chemotherapy of coronavirus infection and provide a critical summary of the development of drugs against COVID-19 from the drug repurposing strategy up to the molecular design of novel covalent and non-covalent agents capable of inhibiting virus replication. We overview the main antiviral strategy and the choice of SARS-CoV-2 Mpro and PLpro proteases as promising targets for pharmacological impact on the coronavirus life cycle.

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1. Introduction

At the end of 2019, an outbreak of severe acute respiratory syndrome (SARS) led to the global epidemic, which was recognized by the World Health Organization (WHO) as a pandemic on March 11, 2020. SARS and other manifestations of the disease were caused by the SARS-CoV-2 virus, previously not circulating in human intercourse, and received the official name – coronavirus disease 2019 (COVID-19).[1]

SARS-CoV-2 affected about 770 million people in 2 years, causing respiratory, renal, cardiac, cerebral and intestinal manifestations of the disease, which ranged from mild to fatal consequences.[2,3] As of June 20, 2023, 2.5 years after the diagnosis of the disease, about 6.95 million people with a confirmed diagnosis of COVID-19 have died.[4]

Extensive vaccination campaigns have shown effectiveness against the COVID-19 pandemic in the EU and worldwide. The total number of administered vaccine doses worldwide amounted to more than 13461.3 million doses. As of January 2023, according to the data presented in the EU, the general improvement of the epidemiological situation with COVID-19 continued. Death rates fell to their lowest level in 12 months. Most countries of the world reported a similar picture. The proportion of patients with COVID-19 in hospitals and intensive care units in the EU was 28% of the maximum recorded levels during the pandemic. However, despite improving the epidemiological situation, COVID-19 continues to burden healthcare systems.

Indicators of excessive mortality (deviation of mortality from the expected level) indicate that the consequences of the disease continue to affect the health of the Earth's population. General statistics show vaccinations do not create lasting immunity against the SARS-CoV-2 virus. The disease COVID-19 continues to spread.[4,5]

On May 5, 2023, WHO stated that COVID-19 is not a Global Public Health Emergency of International Concern (PHEIC). Given that the disease is already well known, it no longer fits the definition of PHEIC, even though it continues. However, this statement does not mean that the pandemic issue itself is over. WHO will develop long-term, ongoing guidance for countries on how to deal with COVID-19 on a continuing basis.[4,5]

Given the continuous evolution of the virus, regular surveillance is currently being carried out under the auspices of WHO in the world, which consists of detecting and monitoring new variants of SARS-CoV-2. The emergence of variants that pose an increased risk to the global health system has prompted WHO to identify specific variants of the virus as those of interest (VOI) and variants of concern (VOC) to set priorities for decision-making on whether vaccines should be updated or maintained or intensified in response to the increased presence of a particular variant of the SARS-CoV-2 virus (Table 1).[5,6]

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Table 1 The main known variants of SARS-CoV-2

Variant of virus SARS-CoV-2	The earliest documented case
Alfa	Great Britain, September 2020
Beta	North African Republic, May 2020
Gamma	Brazil, November 2020
Delta	India, October 2020
Omicron	Several countries, November 2021

Omicron is currently the dominant variant circulating worldwide, accounting for over 98% of viral sequences.[7]

The main problem with vaccines is the continuous mutation of SARS-CoV-2. Most RNA viruses evolve rapidly. SARS-CoV-2 variants with different phenotypic characteristics, including transmissibility, severity, and immune evasion, continue to emerge rapidly. The Omicron variant discovered at the end of November 2021 marked the beginning of a new phase of the pandemic.[8] It is less virulent but more transmissible than earlier variants of SARS-CoV-2.[9] Even at the beginning of the discovery, the Omicron variant included three sister lines (BA.1, BA.2 and BA.3).[8] Unlike the original SARS-CoV-2 virus, which infected bronchial and lung cells, Omicron BA.1 preferred to replicate in the nasopharynx.[10]

SARS-CoV-2, like other RNA viruses, shows a significant degree of antigenic evolution, which opens up a sufficient probability of repeated infections. Omicron, for example, has a much higher ability to cause reinfection than any of the previous options. Examining the current trends of Omicron variants, we have publications to expect new waves of infection every additional approximately four months of virus circulation, and going forward SARS-CoV-2 will have a more regular incidence pattern like that of seasonal influenza, leading to an expectation of two to three times the annual burden the flu.[10] With the emergence of new variants of SARS-CoV-2 that evade the immune response, vaccines developed against the virus may become less active.[8] It means that the issue of the need to develop drugs against COVID-19 continues to be relevant. The importance of the emergence of new therapeutic drugs against coronavirus disease is evident.[11]

2. General characteristics of the virus SARS-CoV-2

According to the classification of the International Committee on Taxonomy of Viruses (International Committee on Taxonomy of Viruses, ICTV), the SARS-CoV-2 virus belongs to β-coronaviruses.[12] The Baltimore classification of viruses is a classification of viruses into groups depending on the type of genomic nucleic acid (DNA, RNA, single-stranded, double-stranded) and the method of its replication.[13] In the Baltimore classification system, the SARS-CoV-2 virus belongs to the IV group of viruses containing single-stranded RNA with positive single-stranded RNA viruses (+ssRNA).[14,15]

Replication of +ssRNA genomes occurs in the cytoplasm of the host cell in parallel with the assembly of the nucleocapsid, in which the genetic material is packaged.[16] The close connection between viral genome replication and nucleocapsid formation means that the genomic RNA and protein components of the virus are highly specific to each other, and the assembly of the +ssRNA virus depends on the interaction between proteins and between proteins and RNA.[17,18] Most of the genomic RNA encodes two replicative polyproteins, which are then cleaved during cleavage into 16 nonstructural proteins by viral proteases.[14,15] The rest of the SARS-CoV-2 genome encodes four structural proteins: spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N).[8,16–19]

3. Basic strategies of antiviral therapy

In the development of antiviral drugs, two different strategies are used: the influence on the cells of the host organism (indirect methods) or the influence directly on the virus (direct methods).

The indirect methods aim to target proteins of the host cell involved in the virus's life cycle. This approach has a lower probability of developing resistance but significantly higher toxicity.[19] Coronaviruses can enter host cells in three ways: receptor-mediated plasma membrane fusion, receptor-mediated endocytosis, or antibody-dependent viral entry. Therefore, receptor proteins on the host cell surface play a crucial role in virus recognizing and attaching host cells for both fusion and endocytosis.[20] Preventing binding interactions between SARS-CoV-2 and human angiotensin-converting enzyme II (ACE2) receptors provides example of this strategy[21] and a foundation for developing new vaccines against COVID-19.[22] Some excellent reviews on this topic has recently appeared.[17,19,20]

The direct methods affect specifically on the certain components of the virus and its life cycle. These approaches are diverse and characterized by significant specificity and fewer side effects for the body. Viral proteins, such as main protease Mpro and papain-like protease PLpro, as well as other components of the virus at different stages of its life cycle can be the potential targets of the antiviral strategy of the direct approach. General therapeutic approaches to the treatment of viral infections, as a rule, are explicitly directed at viral components. However, such drugs are very sensitive to drug resistance due to rapid changes in the viral genome, especially for RNA viruses. When designing molecules for a pharmaceutical effect on the virus, it is necessary to take the most conservative targets: components of the virus that have fewer mutations and are highly specific for the virus. Reducing the risk of developing resistance in developing drugs focused on more than one viral component.[23] This procedure requires particular approaches to the development of antiviral drugs.[19]

In this review, we will focus on research progress in designing and developing directly acting small-molecule inhibitors against COVID-19, focusing on their chemical structure, structure–activity relationship, and clinical treatments.

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4. Life cycle of virus SARS-CoV-2

The life cycle (LC) of the virus includes several main phases:

– Binding.

– Release of the viral genome.

– Genome replication.

– Expression (synthesis) of viral specific structural and non-structural proteins.

– Processing of virion proteins at the maturation stage assembly of the viral capsid.

The first stage of LC is the attachment of the virus to the cell and penetration into the cell. Attachment and penetration can occur by receptor-mediated endocytosis or by direct membrane fusion. With the help of the surface S protein, the SARS-CoV-2 virus attaches to the surface receptor of angiotensin-converting enzyme-2 (ACE2) of the host cell (Fig. 1).[19]

The second stage of LC is the release of the viral genome. The viral genome is released in the cell from the viral particle with the help of specific proteases. In this phase, the virus can use the proteases of the infected cell (host cell).

The third stage of LC is the replication of the virus genome. The virus can use the host's machinery to replicate the viral genome in this phase.

The fourth stage of LC is the expression of viral proteins utilizing translation – the process of protein synthesis on the information RNA matrix, which is carried out by the ribosome of the host cell.

A large part of the genomic RNA of the virus encodes two polyproteins – pp1a and pp1ab. The rest of the genome encodes four structural proteins: S-protein, envelope protein (E), membrane protein (M), nucleocapsid (N) protein and others.[25]

The fifth stage of LC is the stage when the newly formed polyproteins pp1a and pp1ab undergo post-translational modification, so they are proteolyzed (cut) into smaller proteins by two viral proteases, 3C-like protease (3CLpro), also known as main protease (Mpro), and papain-like protease (PLpro). Cleavage products include 16 nonstructural proteins (nsp), including RNA-dependent RNA polymerase (RdRp).[25]

The sixth stage of LC is the stage of assembly (maturation) of the virus: new viral particles are formed, ready to be released from the host cell.[26]

5. Molecular architecture of the virus SARS-CoV-2

The development of potential antiviral drugs requires understanding both the life cycle of the SARS-CoV-2 virus and its molecular architecture. State-of-the-art research in cryo-electron tomography (cryoET) has allowed us to visualize intact SARS-CoV-2 virions and gain insight into their structure in their natural state with a resolution close to atomic resolution.[12]

SARS-CoV-2 virions are approximately spherical or ellipsoidal with an average diameter of 108 ± 8 nm. The outer surface of the virion is covered with surface spike-like proteins (S-protein). The virus's outer membrane contains a membrane protein (M) and an envelope protein (E). The E protein is small, with a mass of 8.5 kDa, forming a membrane channel. The main protein component in the middle of the virion is the nucleocapsid protein N, responsible for binding genomic RNA and packaging it into a ribonucleoprotein (RNP) complex.[12,27]

The genome of CoVs is 27–32 kb (kilobase pairs) and is the second largest of all RNA virus genomes. Almost two-thirds of genomic RNA has two open reading frames (ORFs): ORF1a and ORF1b at the 5′-end, which are quickly translated into two polyproteins – pp1a and pp1ab, which are converted by two viral proteases into sixteen non-structural proteins (nsp1-16).[25] Another third of the genome encodes four structural proteins: spike protein (S), envelope protein (E), membrane protein (M), nucleocapsid protein (N) and other intermediate proteins. ORFs encode these proteins at the 3′-end of genomic RNA.[9,12,28]

6. Structural and non-structural proteins of SARS-CoV-2

6.1. Spike protein (S protein)

The spike surface protein of SARS-CoV-2 (S protein) is a structural transmembrane glycoprotein located on the surface of the

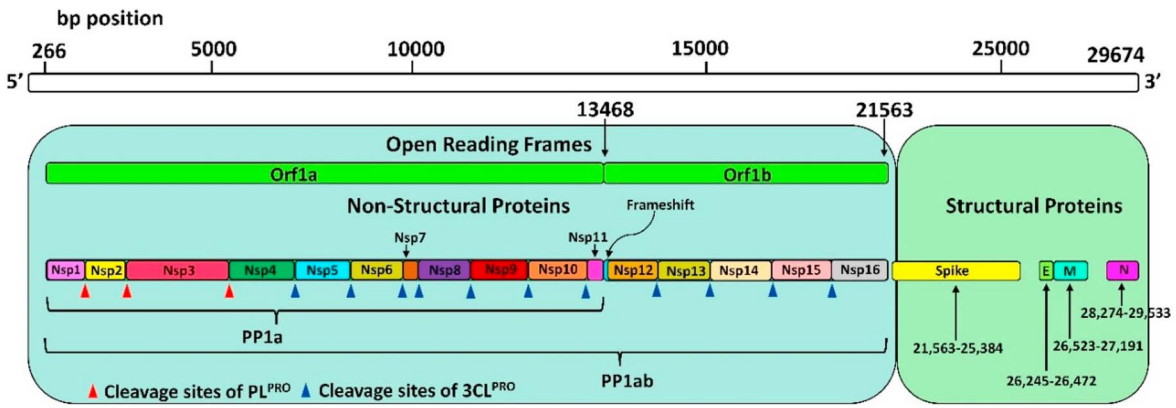

Fig. 1 SARS-CoV-2 genome encoding 5 structural proteins and 16 non-structural proteins (Nsp) translated into a single polyprotein chain (PP1ab). Red (PLpro) and blue (Mpro) triangles show the cleavage sites of the polyprotein chain by proteases. Reproduced with permission from ref. 24. © 2021 Elsevier B.V. All rights reserved.

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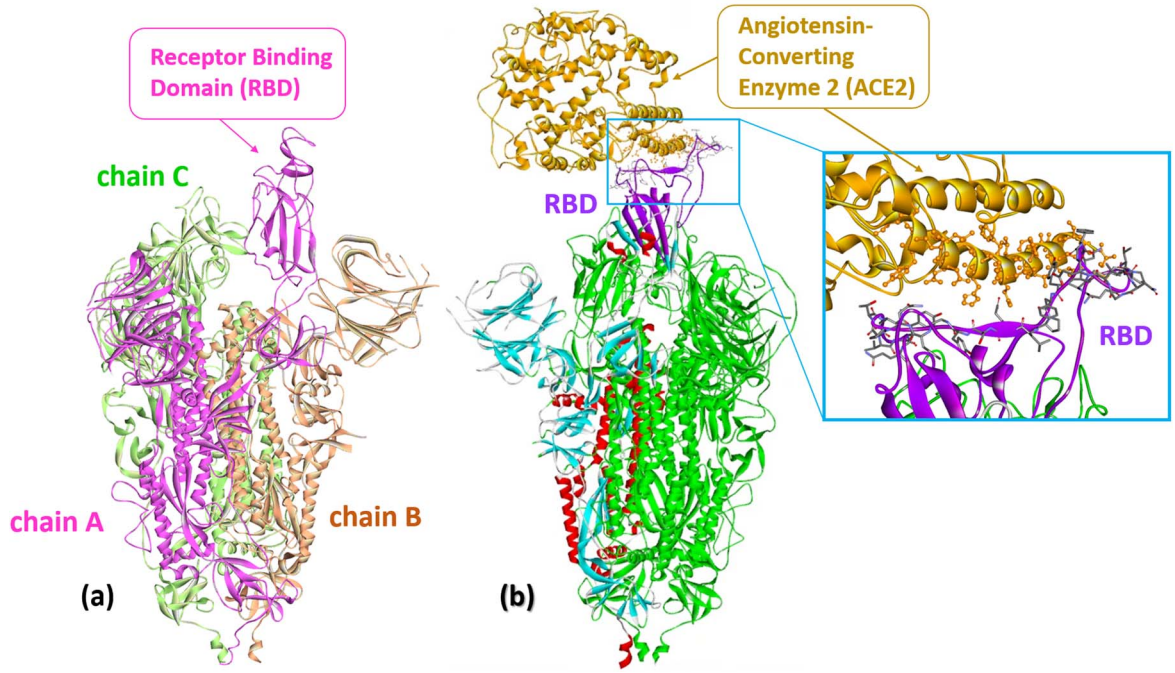

Fig. 2 (a) The S protein of SARS-CoV-2 (Omicron S-open-2, PDB: 7WVO): chains A–C with receptor-binding domain (RBD). (b) Cryo-EM structure of SARS-CoV-2 spike protein in complex with angiotensin-converting enzyme 2 (ACE2) shown in gold (PDB: 8HFZ). A close-up representation of the RBD protein and ACE2 interface. The sticks represent the key residues that interact with the ACE2. Numerous atomic connections are made between SARS-CoV-2 RBD (violet) and ACE2 (gold).

viral particle. It is a trimer with a molecular weight of about 600 kDa (Fig. 2a).[29] The S protein recognizes the receptor of angiotensin-converting enzyme 2 (ACE2) on host cells and, through its receptor-binding domain (RBD), ensures the penetration of the virus into the host cell due to the fusion of the membranes of the virus and the host cell (Fig. 2b).[12,30,31] Because of its key role in progressing SARS-CoV-2 infection, the S protein is one of the main targets for vaccine and drug developers. The S protein is the antigen of all vaccines currently on the market and elicits a strong immune response in vaccinated and infected individuals.[32]

The surface of the S protein is highly glycosylated. Such a glycan shell, combined with the flexibility of the spikes of SARS-CoV-2, allows them to scan the host cell's surface and bind to the cellular receptor ACE2, possibly providing protection against neutralizing antibodies.[29,32] The S protein is a trimer, in which each monomer consists of two subunits: S1 and S2.

The S1 subunit includes an N-terminal domain (NTD), a receptor-binding domain (RBD), and subdomains associated with the RBD and NTD. In the early stages of infection, the S protein undergoes several conformational changes: the closed initial conformation minimizes the antigenic surface of the virus, and the open conformation at the contact stage with the ACE2 receptor has a much larger available antigenic surface.[33] Transitions between open and closed conformations are associated with the opening of the trimer and large movements of the domains, especially the RBD, which are straightened to engage the receptor (Fig. 2a).[32,33]

The crystal structure of the S protein of SARS-CoV-2 has recently been resolved (PDB ID: 2AJF, 6ZGE, 7BNN).[34–36] An example of the S protein of SARS-CoV-2 Omicron (PDB: 7WVO) is shown in Fig. 2a.

The S protein is one of the most immunogenic among other SARS-CoV-2 proteins. The emergence of new Alpha, Beta, Gamma and Delta variants of SARS-CoV-2 was associated with the presence of common S protein mutations in the N-terminal domain (NTD), the receptor-binding domain (RBD) and C-terminal domain (CTD). Mutagenesis studies of SARS-CoV found that two proline substitutions at residues 986 and 987 stabilize the S protein in its pre-fusion form, which elicits a strong immune response.[27]

The current strategies to inactivate the S protein rely on preventing the viral spike protein from binding to the cellular receptor ACE2 (Fig. 2b).[17,22] Considering the allosteric mechanism of RBD-ACE2 binding, it seems that low-molecular weight ligands are not effective in this role.[20] Instead, developing efficient RBD-ACE2 blockers is promising alternative.[21,37,38] In addition, several neutralizing monoclonal antibodies, such as nCoV617 (PDB: 7E3O),[39] B38 and H4 (PDB: 7BZ5)[40] were suggested. The S protein stabilized by this double-proline mutation was used to develop mRNA vaccines mRNA-1273 and BNT162b2 by Moderna and Pfizer-BioNTech in December 2020 and January 2021, respectively.[41,42] Since 2021, the BNT162b2/Pfizer-BioNTech vaccine has been approved in 85 countries from North and South America and Europe, while mRNA-1273/Moderna has been allowed in 45 countries in Europe and North America.[43]

A recent study of structural changes in the S protein in the main variations of SARS-CoV-2 revealed several regularities in the evolutionary dynamics of the virus. Spikes of variants

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acquired amino acid changes linked to increased virulence and immune evasion. In the initial Wuhan variant, the S protein was in a closed form before contact with the ACE2 receptor.[12] Such structure may have provided protection against neutralizing antibodies. During the virus evolution, the ability of the spike of SARS-CoV-2 to switch to an open conformation became a competitive advantage because it facilitated binding to the receptor. At the same time, antibodies targeting the open subdomain of the RBD were rare from the point of view of population immunity.

Alpha-, Beta-, and Delta-variants of SARS-CoV-2 are variations with an open S protein structure. The Omicron variant has an already closed or partially closed conformation of the S protein and hides the RBD epitopes that are now recognized by antibodies due to mass vaccination. As a result, the previously circulating Alpha, Beta, and Delta variants are now non-competitive and disappearing at the population level.[32]

Permanent SARS-CoV-2 mutations are part of the evolutionary development of the virus, and the S protein is its dynamic part that directly responds to the environment and changes. Such responses to changes occurring in the S protein region lead to changes in the immunogenic characteristics of the virus and evasion of the virus from immunity.[44]

Several drugs that are monoclonal antibodies (mAbs) targeting the SARS-CoV-2 adhesion protein have received emergency use authorization (EUA) from the US Food and Drug Administration (bamlanivimab plus etesemab, cazirivimab plus imdevimab, sotrivab and bebtelovimab). However, they are not currently approved for use in the United States because it is expected that the activity of anti-SARS-CoV-2 mAbs against specific variants and subvariants of the virus may vary dramatically. The COVID-19 Treatment Guideline Panel recommends against the use of monoclonal antibodies against SARS-CoV-2 for the treatment or prevention of COVID-19 because the dominant subvariants of Omicron in the United States are not expected to be susceptible to these products.

The rapid evolution of the viral genome, focused mainly on S-protein mutations, creates problems when using the S-protein as a target for drug discovery. Therefore, the use of highly specific and more mutation-resistant targets is necessary for the development of new drugs against SARS-CoV-2.[30]

6.2. Nucleocapsid protein (N protein)

The nucleocapsid protein (N protein) of SARS-CoV-2 plays an important role in the life cycle of the coronavirus. When SARS-CoV-2 enters the host cell, the N protein dissociates from the positive strand (+) of the RNA genome of the virus. After that, the program of replication and expression of the viral genome begins. The N protein is a structurally heterogeneous multidomain RNA-binding protein with a length of 419 amino acids, which has the ability to recognize and bind RNA. The N protein is responsible for the identification and packaging of viral RNA. It binds to the genomic RNA of the virus, forming a ribonucleoprotein complex (RNP). The SARS-CoV-2 N protein consists of three highly disordered regions: the N-arm, the central linker region (LKR), and the C-tail, as well as two structural domains:

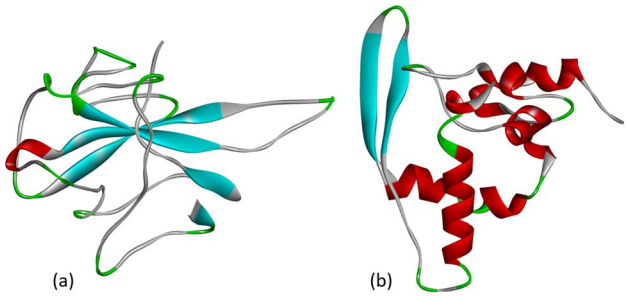

Fig. 3 (a) Crystal structure of the N-terminal RNA-binding domain of the nucleocapsid protein (PDB: 6M3M) and (b) the C-terminal dimerization domain of the SARS-CoV-2 nucleocapsid (PDB: 6ZCO).

the N-terminal RNA-binding domain (NTD) and the C-terminal dimerization domain (CTD).[27] The crystal structure of the NTD domain and the C-terminal dimerization domain of the SARS-CoV-2 nucleocapsid has currently been characterized as shown in Fig. 3.

Because the RNA-binding activity of the N protein is critical for the formation of viral particles, blocking RNA binding is one strategy for therapeutic effects, and N proteins are attractive targets for antiviral drugs.[45] Contrary to the spike protein, the structure of which has been affected by mutations, such as Alpha, Beta, Gamma, Delta, Epsilon, and Omicron emerged in a period of the last two years, the N protein sequence alignments revealed ~98% conserving.[45] Since the beginning of the pandemic, some *in silico* studies have been conducted to identify potential SARS-CoV-2 nucleocapsid inhibitors using molecular docking-based drug repurposing.[46,47] At the same time, such research did not lead to the development of drugs of such a focus. One of the reasons for the failure of developing small-molecule therapeutics based on nucleocapsid proteins might be the lack of well-defined binding and catalytic sites.

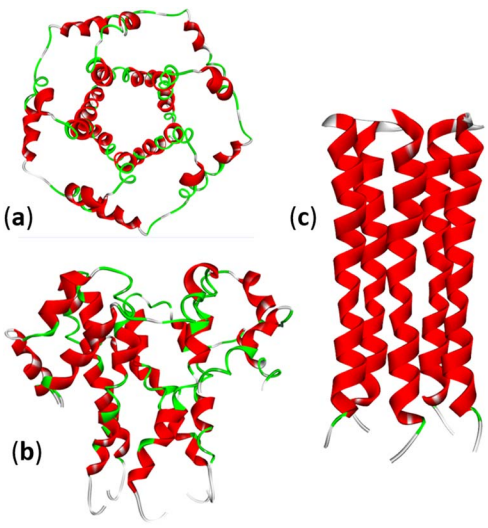

Fig. 4 Structure of the SARS-CoV-2 E-protein pentameric ion channel (PDB: 5X29): (a) top and (b) side views. (c) The pentameric structure of the transmembrane domain of the E-protein of SARS-CoV-2: (PDB: 7K3G).

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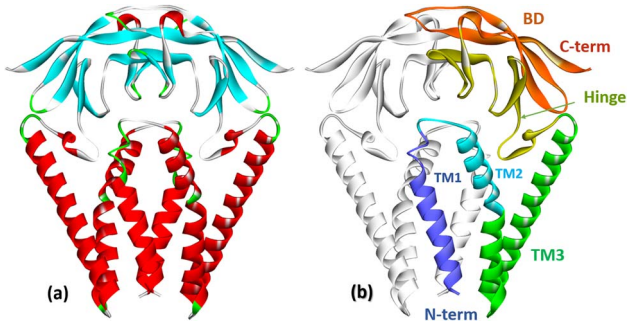

Fig. 5 (a) Crystal structure of the membrane protein (M-protein) of the coronavirus SARS-COV-2 (PDB: 8CTK). (b) Structural components of the M protein: two transmembrane three-helical bundles (TM1, TM2, TM3) and two intravirion Hinge domains and C-terminal β-sheet sandwich domain (BD).[50]

The N protein plays various important roles in the life cycle of SARS-CoV-2, not only related to the formation of a ribonucleoprotein complex with viral RNA. Further studies of the interaction between SARS-CoV-2 N protein and other viral and host cell proteins under disease conditions are necessary to understand the molecular mechanisms of the effect on the SARS-CoV-2 nucleocapsid.[48]

6.3. Envelope protein (E protein)

The envelope CoVs protein (E protein) is a small transmembrane protein with 75–109 amino acid residues and a mass of 8–12 kDa, present in small amounts in viral particles. The E protein can homooligomerize to form a cation-selective ion channel (IC) or viroporin (Fig. 4). Many viruses express viroporins; for example, the M2 protein of influenza A. Amantadine is a broad-spectrum viroporin inhibitor previously used to treat influenza A. Amantadine was tested against SARS-CoV-2, but it showed only moderate inhibition of the E protein activity.[49]

The use of the E protein as a target for pharmacologic effects on SARS-CoV-2 is not currently being actively considered, but it may be a comprehensive strategy to reduce the emergence of resistance, since the virus is less likely to mutate in the part of the genome that encodes this protein.[23]

6.4. Membrane protein (M protein)

The membrane protein (M protein) is the most abundant protein in the envelope, which directs the process of virion assembly through interaction with other structural proteins. The M protein consists of 221 amino acid residues and has three structural components: the N-terminal part protruding from the membrane; transmembrane domain; the C-terminal part is the final part, which includes two domains (Fig. 5). The M protein forms a mushroom-shaped dimer consisting of two transmembrane three-helical bundles and two intravirion domains (Fig. 5).[50]

The M protein can interact with other structural proteins: the interaction of the M protein with the nucleocapsid (N) protein contributes to the stability of the N protein. The M protein directs the process of viral particle assembly by interacting with other structural proteins.[30] It is assumed that the M-protein acts as a framework for the joint assembly of a complex of structural and auxiliary proteins that form both the viral envelope and the viral particle itself.

Taking into account that the M protein is the most abundant protein in the viral envelope, it can be a target for vaccines and therapeutics.[51] However, despite the broad abundance, drugs and potential inhibitors for the M protein have not yet been proposed.

Table 2 Non-structural proteins of SARS-CoV-2 and their functions[52]

Non-structural proteins of SARS-CoV-2	Functions
Nsp1	Ensures RNA processing and replication (PDB: 6ZOK, 2GDT)
Nsp2	Modulates the host cell survival signalling pathway (PDB: 7EXM)
Nsp3	Separates proteins that are translated (PLpro) (PDB: 6W02, 5Y3E)
Nsp4	Contains a transmembrane domain (TM2) and modifies membranes
Nsp5	The main protease (Mpro), participates in the process of cleavage of polyprotein during replication (PDB: 1WOF, 1Z1J, 2C3C)
Nsp6	Is the transmembrane domain
Nsp7	Has an effect on RNA-dependent RNA polymerase (PDB: 1YSY)
Nsp8	The presence of nsp7, nsp8, and nsp11 affects RNA-dependent RNA polymerase and coronavirus replication/transcription
Nsp9	Functioning as an ssRNA-binding protein
Nsp10	Is crucial for cap-methylation of viral mRNAs (PDB: 2G9T)
Nsp11	Together with nsp7, nsp8 targets RNA-dependent RNA polymerase and coronavirus replication/transcription
Nsp12	Contains RNA-dependent RNA polymerase (RdRp), which is a critical component of coronavirus replication/transcription
Nsp13	The zinc-binding domain of nsp13 is involved in replication and transcription processes (PDB: 6XEZ, 7EIZ)
Nsp14	Proofreading exoribonuclease domain
Nsp15	Has Mn^{2+}-dependent endoribonuclease activity (PDB: 6VWW)
Nsp16	2′-*O*-ribosemethyltransferase (PDB: 6WKS)

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6.5. Non-structural proteins

The general genome organization of SARS-CoV-2 includes sixteen non-structural proteins Nsp1–16, which are highly conserved among coronaviruses (Table 2).[30] They are encoded by ORF1a/b at the 5′-end and are expressed as a single polyprotein chain Pp1a and Pp1ab, which is further cleaved into individual proteins by internal proteases (Fig. 1). The SARS-CoV-2 genome encodes two different cysteine proteases that are required for the viral proliferation cycle: papain-like protease (PLpro, domain in Nsp3, EC 3.4.22.46) and chymotrypsin-like main protease (3CLpro or Mpro, domain in Nsp5, EC 3.4.22.69). Viral nonstructural proteins are responsible for virulence and survival of the virus. To perform their function, they must be proteolytically separated from the polyprotein chain, thus proteolysis by two viral proteases plays a critical role in the life cycle of SARS-CoV-2.[14]

Both structural and nonstructural proteins play important roles during different stages of the SARS-CoV-2 life cycle and can be selected as targets for chemotherapy.[30] Along with developing small molecule antivirals, significant research progress has been made toward targeting SARS-CoV-2 RNA-dependent RNA polymerase (RdRp), main protease (Mpro or 3CLpro), and papain-like protease (PLpro).[53]

7. Promising targets for pharmacological action on SARS-CoV-2

7.1. RNA-dependent RNA polymerase (RdRp)

Upon SARS-CoV-2 virus replication, the replication/transcription complex consists of the viral nonstructural proteins nsp7, nsp8, and nsp12.[54] The core of the complex is sp12-RNA-dependent RNA polymerase (RdRp). Nsp12 is weakly active by itself. Some studies demonstrated that the presence of nsp7 and nsp8 significantly increased the combination of nsp12 and template RNA. The crystal structure of the nsp12–nsp7–nsp8 complex was recently identified fpr isolated protein and its complex with remdesevir, as shown in Fig. 6.

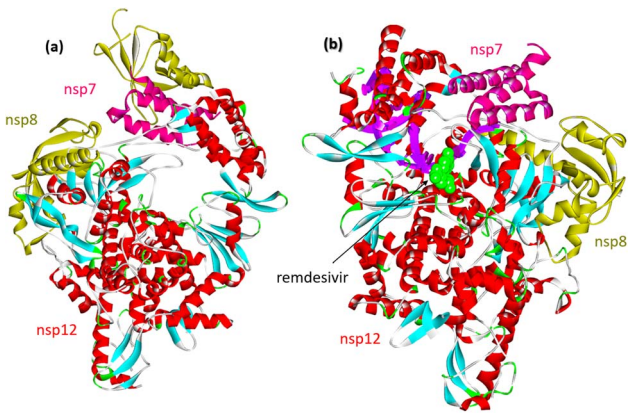

Fig. 6 (a) Cryo-electron microscope structure of the nsp12–nsp7–nsp8 complex (PDB: 7BV1). (b) The nsp12–nsp7–nsp8 complex associated with the template primer RNA and remdesivir triphosphate (RTP) (PDB: 7BV2).

RNA-dependent RNA polymerase, which catalyzes the synthesis of viral RNA, is a critical component of coronavirus replication/transcription. Therefore, RdRp is believed to be an essential target for developing new antiviral drugs.[52,54–58] The first reported inhibitor of SARS-CoV-2 RdRp is Remdesivir, which was identified by a drug repurposing approach (Fig. 7). Remdesivir is a nucleoside analog that inhibits RNA polymerases. Nucleoside analogues usually target viral replication, especially viral DNA or RNA polymerase, and are successfully used in clinical practice in the treatment of viral infections.[58,59] In several studies, the RdRp protein was used as a target receptor for the *in silico* screening for new antiviral drugs.[60–66]

The mechanism of action of drugs belonging to the nucleoside analogues is based on structural similarity with natural nucleosides/nucleotides.[67] However, they have some minor changes in the structure, which are critical for the further fate of the macromolecules in which they are included. Nucleoside analogues are incorporated into the RNA chain synthesized by the virus during replication and disrupt its further assembly.[61] It leads to premature chain termination and, hence, the inhibition of virus replication.[67] A nucleoside analog is defined as a nitrogenous base attached to a sugar where the nitrogenous base or sugar component is altered in such a way that the molecule is different from that found in nature. Modifications of the nitrogenous bases include halogenation, addition of azido groups, and modifications of the sugar component include ring opening, halogenation, methylation, hydroxylation, or dihydroxylation.[68] A number of nucleoside analogues have already passed clinical trials for the treatment of COVID-19 and are on the market. The most successful examples are Remdesivir, Favipiravir, Molnupiravir and Galidesivir (Fig. 7).[56,69]

Remdesivir has been approved by the USA Food and Drug Administration (FDA) as the first small molecule for an "emergency use permit" for the treatment of COVID-19.[70] The full FDA approval was granted in October 2020. Intravenous therapy with this drug is allowed for use in both hospitalized and non-hospitalized patients.[70]

Remdesivir targets RNA-dependent RNA polymerase, which creates new copies of coronavirus RNA.[54,65] Remdesivir mimics the adenine nucleotide, and RNA-dependent RNA polymerase inserts it into the growing RNA molecule instead of adenine.[71] This disrupts the normal form of the RNA chain and interrupts its synthesis. In several clinical trials, treatment of COVID-19 with remdesivir resulted in improved health.[72] A side-effect is, however, that remdesivir is unstable in the blood and has a limited half-life of less than an hour in humans, requiring a continuous infusion.[73]

Favipiravir was originally developed against the flu. It belongs to nucleoside analogues and blocks the ability of the virus to copy its genetic material. It is metabolized in cells to favipiravir ribosyltriphosphate (favipiravir RTF) and selectively inhibits RNA-dependent RNA polymerase.

There are evidences that favipiravir may be effective in SARS-CoV-2 infection. At the same time, the WHO, Great Britain, the

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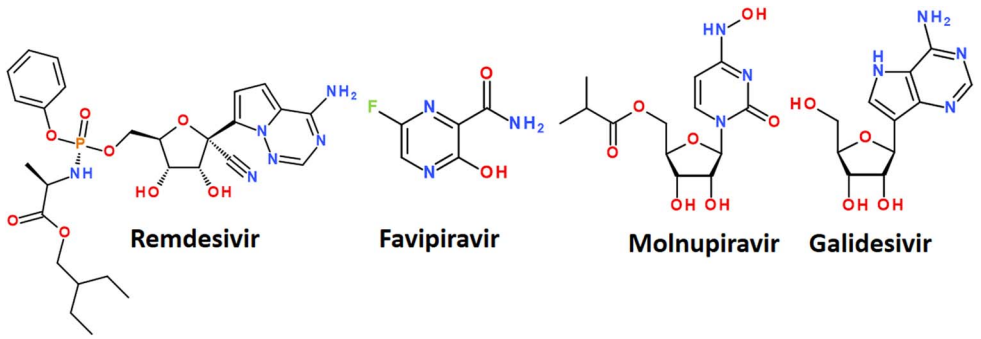

Fig. 7 Some nucleoside analogs available for the treatment of COVID-19.

United States and Canada did not provide recommendations for the use of favipiravir against COVID-19. Some molecular docking studies showed a high affinity of favipiravir to the main Mpro protease of SARS-CoV-2.[74,75]

Molnupiravir is a nucleoside analogue proposed for oral use in the treatment of early stages of COVID-19. Like favipiravir, it was originally developed as an antiviral drug to treat influenza. The mechanism of action is the accumulation of errors in the viral genome, when the drug is incorporated into the RNA of the virus, causes mutations in the viral RNA and thus stops the replication of the virus. In some countries, the drug has been approved for the treatment of confirmed COVID-19 infections.[76]

Galidesivir (Galidisvir, BCX4430) is a novel synthetic adenosine analogue, which effects on the inhibition of RNA transcriptional activity was first assessed in a cell-free isolated HCV RdRp assay.[77] Galidesivir acts as a nonobligate RNA chain terminator[56] and can be administered by both parenteral and oral routes.

A side effect of nucleoside analogues, which limits their clinical treatments, is its ability for disrupting RNA polymerase chains, causing some risks of RNA mutagenesis impairing the human genes.[78]

Finally, natural and synthetic agents, and the most promising repurposed drugs against SARS-CoV-2 RdRp have recently been reviewed elsewhere.[79,80]

7.2. Main protease Mpro

Protein kinases are a class of enzyme-kinases that modify specific proteins and are responsible for the activation of various proteins in the cascade of cellular signaling pathways. Due to the significant effect of protein kinases on cell functioning, they are attractive targets for drug development. There are a significant number of available medicinal substances that are inhibitors of protein kinases, and they were primarily investigated as possible therapeutic agents in the treatment of COVID-19.[11] Some inhibitors of tyrosine kinase tested by the drug-repurposing algorithm showed activity against COVID-19, such as Baricitinib, Fedratinib, Gilteritinib, Imatinib, Ivermectin, Lonafarnib, Nilotinib, Osimertinib, Ruxolitinib, and Tofacitinib, however, their efficacy was insufficient for direct therapeutic use.[81–83]

The main attention of the researchers was focused on the main protease of SARS-CoV-2. Since no known human protease recognizes this cleavage site, Mpro is considered an ideal target for therapeutic development.[27,84,85] In addition, Mpro active sites are highly conserved among all coronaviruses.[52]

Nonstructural protein 5 (Nsp5) or main protease Mpro (3C-like protease) is responsible for the cleavage of eleven Mpro-specific sites on the two SARS-CoV-2 polyproteins Pp1a and Pp1ab into 11 nonstructural proteins (nsp4-16) (Fig. 1). MPro consists of three domains: chymotrypsin-like domain I and 3C-protease-like domain II, as well as domain III consisting of 5 α-helices. The MPro binding pocket is located between domains I and II and has a catalytic CysHis dyad (Cys145 and His41). Cysteine sulfur serves as a nucleophile, and the imidazole ring of histidine serves as a general base. MPro targets the recognition sequence Leu–Gln↓(Ser, Ala, Gly)↓ (where ↓ means a cleavage site) (Fig. 8).[86]

The SARS-CoV-2 Mpro structure consists of three domains: domain I (residues 8–101), domain II (residues 102–184), and domain III (residues 201–303). The third domain is connected to domain II by a long loop region (residues 185–200) (Fig. 9).[87]

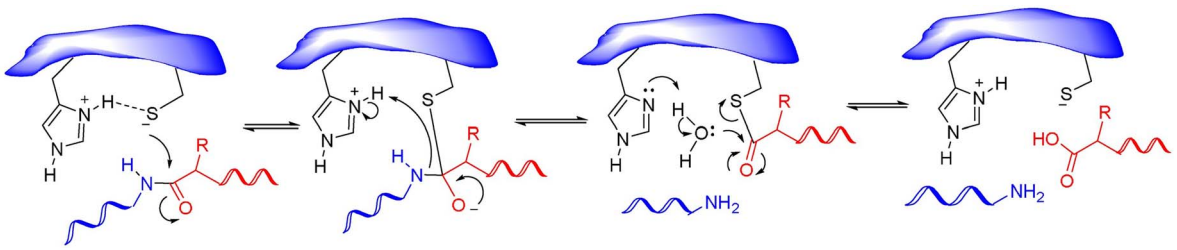

Fig. 8 Mechanism of action of cysteine proteases. Reproduced with permission from ref. 88. © 2020 Elsevier Ltd. All rights reserved.

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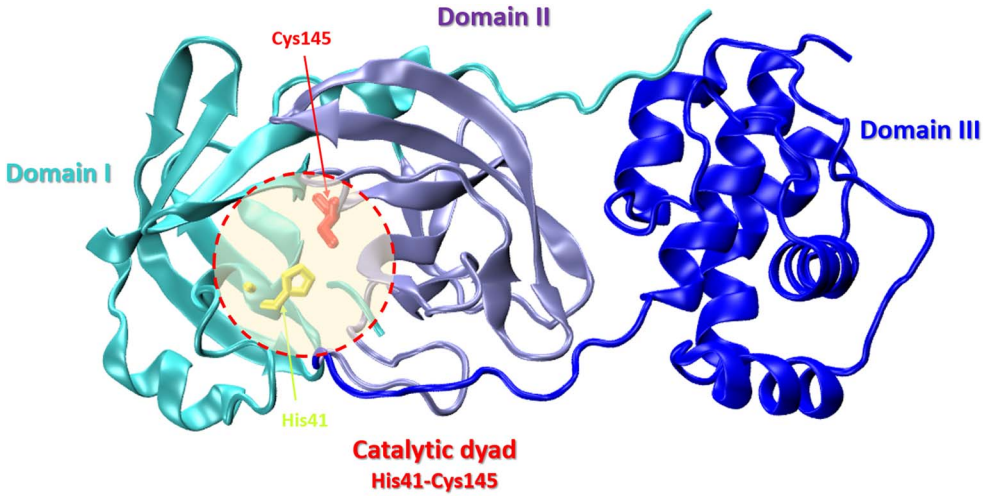

Fig. 9 Crystal structure of SARS-CoV-2 main protease (Mpro) with the catalytic center (His41 and Cys145) (PDB: 6LU7): domain I – cyan, domain II – violet, domain III – blue.

The high-resolution X-ray structures of the Mpro coronavirus protease have been used in many studies to develop antiviral drug candidates targeting SARS-CoV-2.[87,89,90]

Numerous experimental and theoretical studies have been devoted to design and development of novel noncovalent inhibitors of Mpro enzyme.[47,56,84,91–96]

Phytochemical extracts, medicinal plants and aromatic herbs, composed of polyphenols, terpinols, flavonols and their glicosides, have revealed a broad spectrum of inhibitory potency and were suggested as potential anti-COVID agents.[80,97–103] Several comprehensive reviews and updates on recent development of Mpro inhibitors have been released.[104,105] Here we briefly summarize most crucial achievements in this field.

Popular strategies for rational design and discovery of novel inhibitors are often based on re-design, hit-to-lead optimization and hit expansion of available most effective inhibitors.[106] Recently, redesigning of know inhibitor perampanel was guided by free energy perturbation MD simulations, kinetic assays and high-resolution X-ray analysis (PDB ID: 7M8M, 7M8N, 7M8O), resulting in a series of noncovalent, nonpeptidic inhibitors with IC_{50} values in a range of 20–40 nM, as summarized in Fig. 10.[107–109]

Developing noncovalent inhibitors of a coronavirus 3CLpro in the aftermath of the SARS-CoV-1 outbreak, led to the discovery of inhibitor ML300, which also revealed high activity against SARS-CoV-2.[110] Starting from the compound ML300, a structure-based optimization was performed against the SARS-CoV-2 Mpro protease using X-ray analysis of Mpro enzymes in complex with multiple ML300-based inhibitors, including the original probe ML300, resulting in discovery of

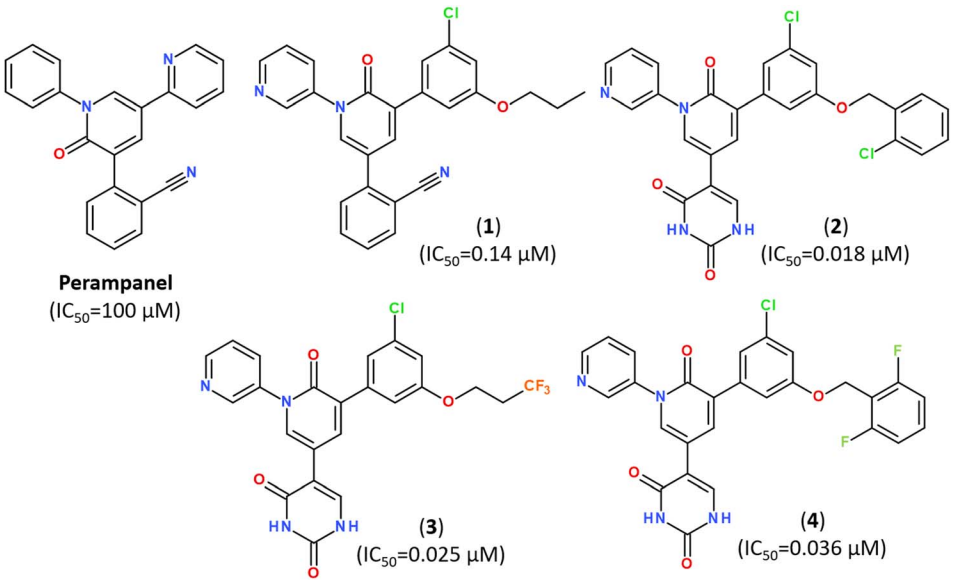

Fig. 10 Structure of Perampanel analogs inhibiting the activity of Mpro.[108]

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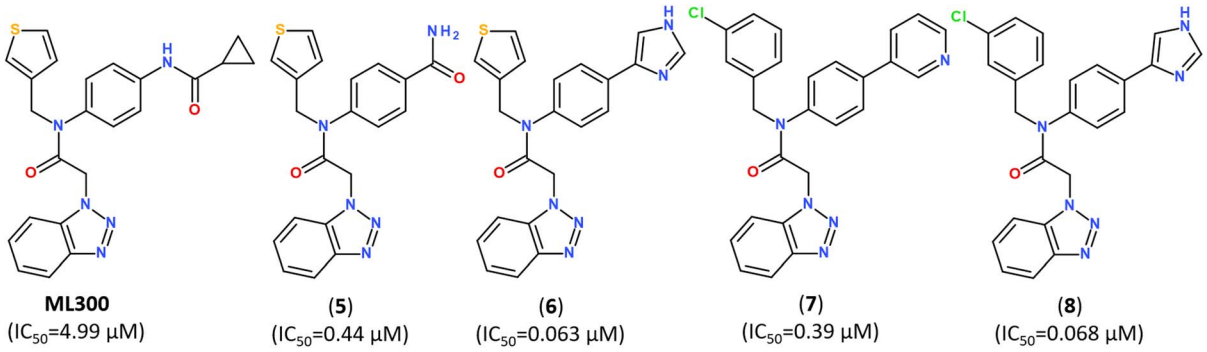

Fig. 11 Structure of noncovalent Mpro inhibitors based on ML300 scaffold.[111]

a series of potent inhibitors with nanomolar IC_{50} values, as summarized in Fig. 11. The X-ray structures of their complexes with Mpro revealed that the disclosed inhibitors utilize a non-covalent mode of action and noncanonical binding mode (PDB ID: 7MLD, 7MLE, 7MLF).[111]

Small-molecule inhibitor MCULE-5948770040 was first identified by scalable high-throughput virtual screening of a targeted compound library of over 6.5 million molecules (Fig. 12).[112] A binding mode of newly-discovered inhibitor was validated by using room-temperature X-ray crystallography (PDB: 7LTJ) and biochemical assays estimated its inhibitory activity with a moderate inhibition constant IC_{50} of 4.2 μM (Fig. 12).

Keeping a 1,2,4-trisubstituted piperazine scaffold, further structure-based rational design was performed and led to the discovery of a series of new potent non-covalent inhibitors, among which GB-13S and GC-14S displayed excellent target selectivity for SARS-CoV-2 Mpro, high antiviral activity, low cytotoxicity (CC_{50} > 100 μM). Moreover, their X-ray co-crystal structures (PDB ID: 8ACL, 8ACD) proved that these inhibitors occupy multiple sub-pockets by critical non-covalent interactions.[93] Recently, novel noncovalent nonpeptidic inhibitor S-217622 (Ensitrelvir) has been discovered *via* a structure-based drug design (SBDD) strategy, starting from virtual screening and followed by X-ray analysis (PDB ID: 7VTH, 7VU6) and biological evaluation of an in-house compound library (Fig. 13).[113]

The compound S-217622 exhibited high antiviral activity *in vitro* against all currently outbreaking SARS-CoV-2 variants, including Delta and Omicron, and showed favorable pharmacokinetic profiles *in vivo* for once-daily oral dosing, which makes it an universal broad-spectrum antiviral clinical candidate for treating COVID-19.[113–115]

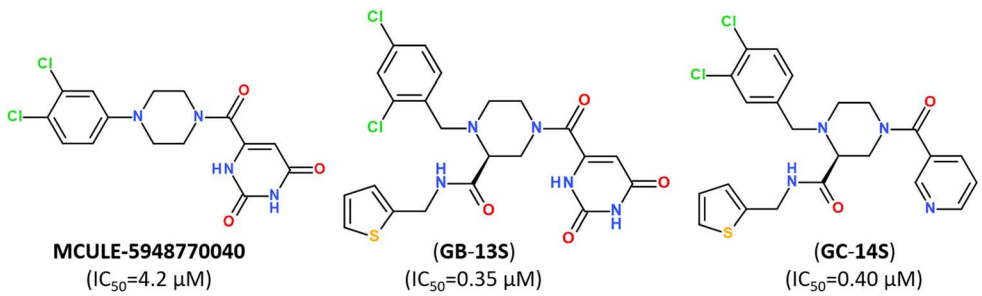

Fig. 12 Structure of non-covalent inhibitor MCULE-5948770040 and its optimized hits.[93]

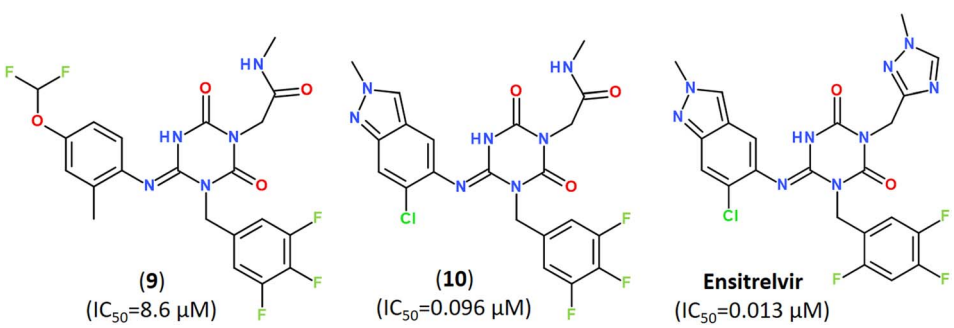

Fig. 13 A noncovalent orally active agent Ensitrelvir (S-217622) and its analogs with strong broad-spectrum anticoronaviral activities.[113]

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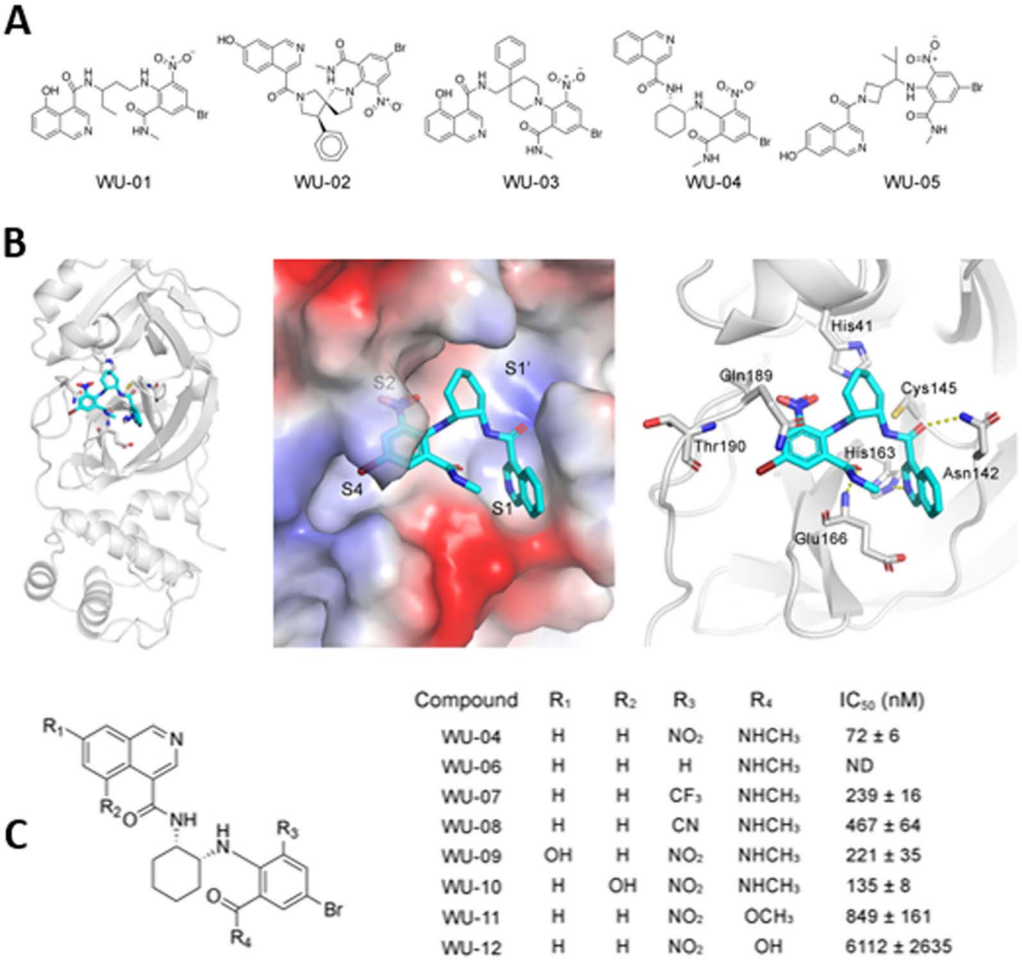

Compound	R_1	R_2	R_3	R_4	IC_{50} (nM)
WU-04	H	H	NO_2	$NHCH_3$	72 ± 6
WU-06	H	H	H	$NHCH_3$	ND
WU-07	H	H	CF_3	$NHCH_3$	239 ± 16
WU-08	H	H	CN	$NHCH_3$	467 ± 64
WU-09	OH	H	NO_2	$NHCH_3$	221 ± 35
WU-10	H	OH	NO_2	$NHCH_3$	135 ± 8
WU-11	H	H	NO_2	OCH_3	849 ± 161
WU-12	H	H	NO_2	OH	6112 ± 2635

Fig. 14 (A) Structure of noncovalent inhibitors of SARS-CoV-2 Mpro, containing an isoquinoline ring and a bromophenyl ring. (B) The binding of noncovalent inhibitor WU-04 into the catalytic pocket of SARS-CoV-2 Mpro. (C) Seven hit analogs of WU-04 and their inhibitory activity against SARS-CoV-2 Mpro evaluated using the fluorescent assay. Adapted with permission from ref. 96. Copyright © 2023 The Authors. Published by American Chemical Society.

A series of specific, noncovalent inhibitors of Mpro, capable of effectively inhibiting SARS-CoV-2 replications in human cells with EC_{50} values in the 10 nM range, has been reported.[96] WU-04 also inhibits the Mpro of SARS-CoV and MERS-CoV with high potency, similar to that of PF-07321332 (Nirmatrelvir) (Fig. 14).

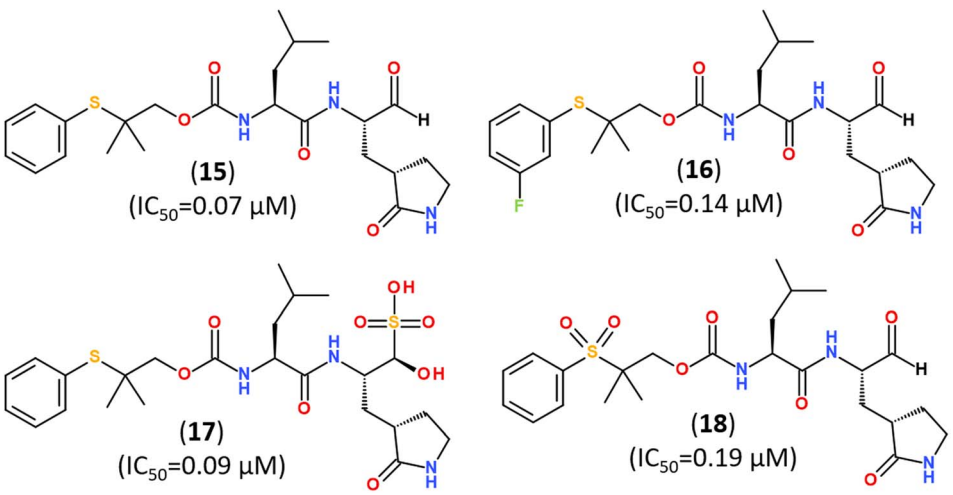

Fig. 15 Direct-acting covalent inhibitors of Mpro that exploited the gem-dimethyl effect.[116]

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Several promising strategies for developing Mpro inhibitors utilize peptidomimetic scaffolds.[83,117–122] A structure-guided design of direct-acting antivirals that exploited the gem-dimethyl effect and inhibited protease Mpro allowed identifying a series of highly potent covalent inhibitors shown in Fig. 15. They inhibited both SARS-CoV-2 and MERS-CoV 3CL proteases in biochemical and cell-based assays. The binding mode of novel inhibitors was confirmed by the X-ray analysis (PDB ID: 8F44, 8E5X).[116]

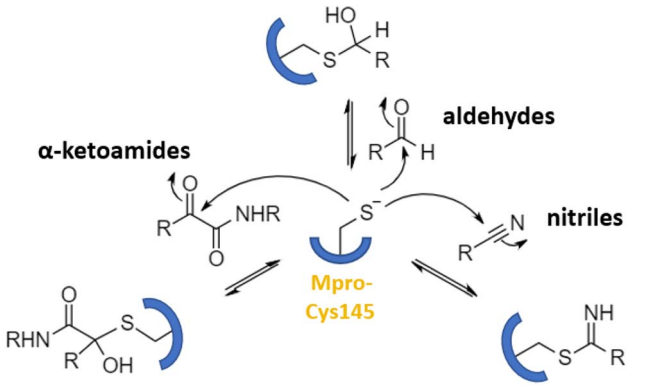

Fig. 16 Different mechanisms of covalent Mpro inhibition, involving α-ketoamides, aldehydes, and nitriles. Reproduced with permission from ref. 104. © 2023 by the authors. Licensee MDPI, Basel, Switzerland.

Numerous covalent-acting peptide-like inhibitors of Mpro protease have been discovered and characterized.[123–129] Covalent inhibitors possess a reactive functional moiety that forms a temporary covalent bond upon reaction with the nucleophilic cysteine thiolate. Popular reactive groups consist of aldehydes and ketones form hemithioacetals, and nitriles form a reversible thioimidate (Fig. 16).[104] Some of covalent Mpro inhibitors are summarized in Fig. 17.

While some FDA-approved HCV drugs, such as Narlaprevir and Boceprevir, have revealed high potency against SARS-CoV-2 too,[120,123,130,131] their clinical use was not reported yet (Fig. 18). Currently, Nirmatrelvir (PF-07321332) is only 3CLpro protease inhibitor approved for medical use against SARS-CoV-2 (Fig. 18).[132–134] Nirmatrelvir in combination with ritonavir is available as the combination antiviral drug "Paxlovid" for oral use, developed by Pfizer.[135,136] Ritonavir is believed to have a synergistic effect on antiviral activity.[137,138]

Nirmatrelvir is a reversible covalent inhibitor carrying a nitrile warhead that binds to the 3C-like protease of SARS-CoV-2, interrupting the viral replication cycle.[90,123,139,140] It has been reported that Nirmatrelvir demonstrated distinct antiviral potency against prevalent variants of SARS-CoV-2, such as Alpha, Beta, Delta, Gamma, Omicron[141] and different human

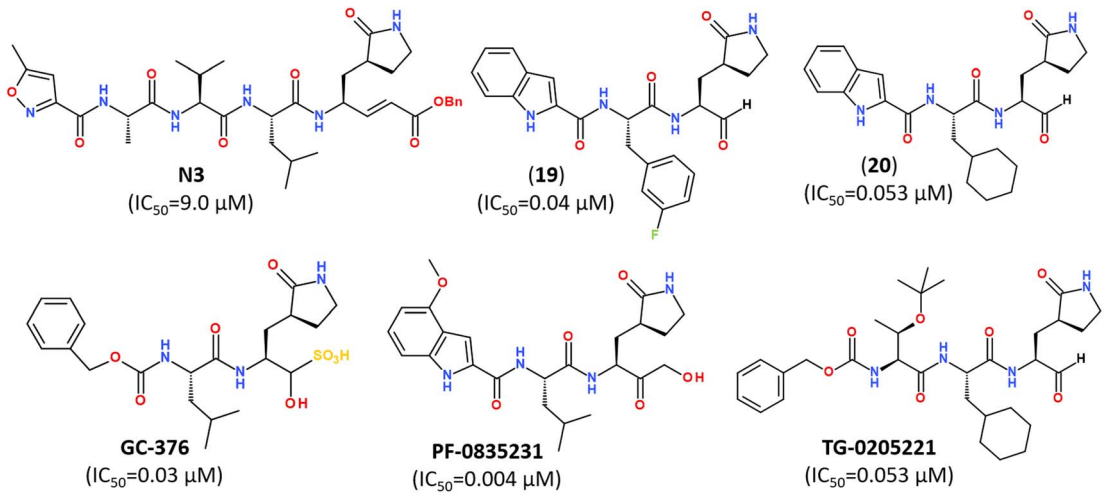

Fig. 17 Reported peptidomimetic Mpro inhibitors with the high inhibitory activities (μM).[120]

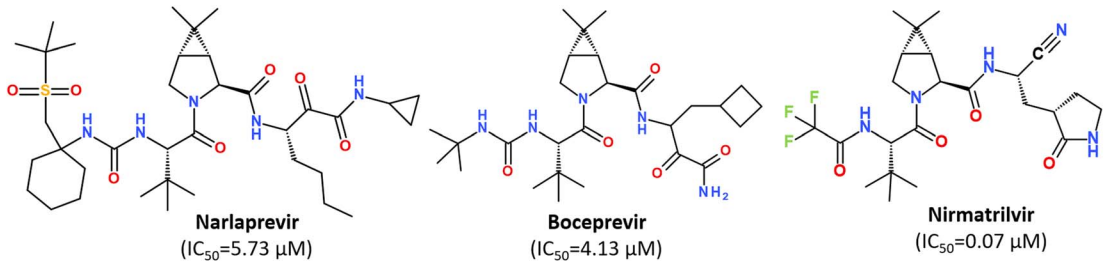

Fig. 18 Chemical structure of Narlaprevir, Boceprevir and Nirmatrelvir.[120]

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coronaviruses.[142] Nirmatrelvir has become a promising scaffold for computer-guided design of its better-acting analogs.[143] Using a hybrid approach combining machine learning and free energy simulations, six new derivatives were designed by modifying parent nirmatrelvir, so that they bind strongly to SARS-CoV-2 Mpro and might be less toxic to the human body than the original inhibitor.[144]

7.3. Papain-like protease PLpro

Papain-like protease (PLpro) is a 35 kDa domain of Nsp3, part of a 215 kDa multidomain protein.[145] This protease cleaves the peptide bonds between Nsp1 and Nsp2, Nsp2 and Nsp3, and Nsp3 and Nsp4, releasing three proteins: Nsp1, Nsp2, and Nsp3. In addition, PLpro is involved in counteracting the host's immune response during viral infection.[146] The SARS-CoV-2 papain-like protease PLpro has long been recognized as a critical enzyme in the virus's life cycle.[8]

It is highly conserved, found in all coronaviruses, and has low sequence similarity with human enzymes.[8] All these features make it a promising target for antiviral drugs.[147] Some studies of PLpro led to the identification of many inhibitors specific to SARS-CoV PLpro.[53] Moreover, both non-covalent and covalent inhibitors of PLpro have been discovered.[56,148–151]

The SARS-CoV-2 PLpro is a protein composed of 315 amino acid residues, with a high content of cysteine residues (3.5%). Several high-resolution structures of free PLpro and its complex with inhibitors have become available (PDB: 7JIR, 7CMD, 6WX4, 6WUU, 6XA9, 6XAA).[147,152–154] The PLpro structure contains three domains: a small N-terminal ubiquitin-like (Ubl) domain, a zinc-binding domain, and a catalytic thumb-palm domain, which is sometimes considered as two separate domains (Fig. 19). The zinc-binding domain, which includes four cysteines, coordinates the Zn^{2+} structural cation in the finger subdomain.[53] This domain is believed to maintain the structural integrity of the whole PLpro protein.[155]

It should be noted that the N-terminal ubiquitin-like (Ubl) domain of PLpro is not specific and has similarities with human cellular DUBs (DUBs are proteases that cleave ubiquitin chains from protein substrates). Ubl inhibitors can affect human DUBs and lead to side effects. For example, Otubain-1 and Otubain-2 are PLpro-like cysteine proteases with the same triad of catalytic Cys/His/Asp, Asn residues.[156] This issue might be one of the possible reasons why essential efforts in developing PLpro inhibitors have not yet led to the development of FDA-approved drugs for therapeutic effect on SARS-CoV-2 in humans.

The catalytically active site of cysteine cleavage (also called the active center) is located between the "thumb" – "palm" domains and contains the canonical catalytic triad for cysteine proteases Cys111-His272-Asp286 (ref. 147) (Fig. 19), which recognizes the sequence of Leu-X-Gly-Gly residues found in-between viral proteins nsp1 and nsp2, nsp2 and nsp3, and nsp3 and nsp4 (nsp1/2, nsp2/3, and nsp3/4) and performs their proteolysis – cleaves them after the second Gly.[147,157] Of all PLpro cysteines, Cys111 is the most susceptible to oxidation, indicating its unique reactivity toward electrophiles.[145] PLpro binds to viral proteins through its active site to fulfill its biological role. The catalytically active site of PLpro is a conservative drug target among SARS-CoV-2 variants. Although some mutations have been identified, all most common mutations are located distal to the binding site.[53,158]

As mentioned above, from a structural point of view, PLpro seems to be a less attractive target for drug development compared to Mpro because it shares structural similarities with human DUBs. Nevertheless, the growing number of recent studies on PLpro inhibitors indicate that it remains one of the promising targets for the development of small molecule therapeutics against SARS-CoV-2.[145,155,159]

Covalent peptide inhibitors VIR250 and VIR251, consisting of a Gly–Gly mimetic linker and a reactive electrophile, have proved to be active and selectively inhibited the PLpro of SARS-

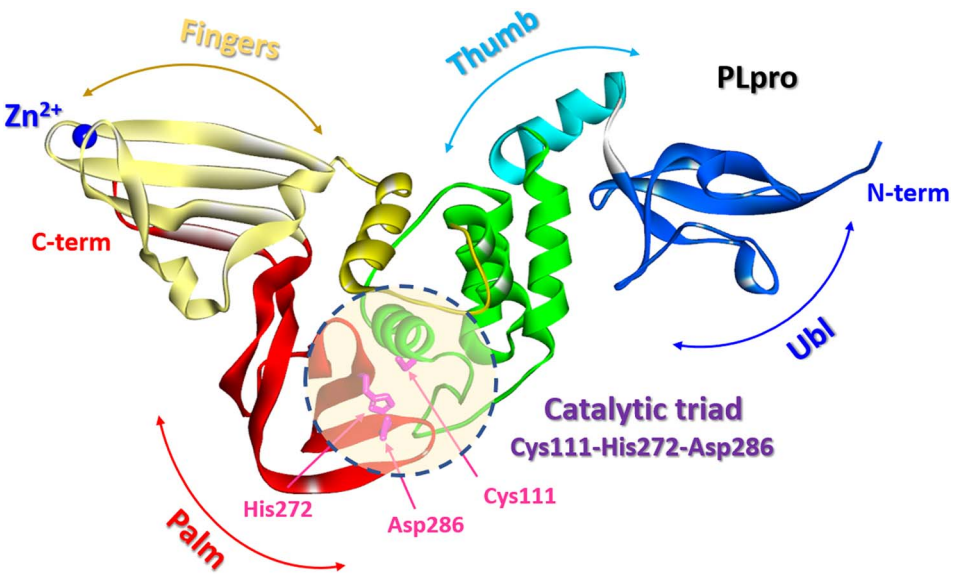

Fig. 19 PLpro structure: terminal ubiquitin-like (Ubl) domain, zinc-binding domain, and catalytic thumb-palm domain. The catalytic active site containing the catalytic triad Cys111-His272-Asp286 (shaded circle) (PDB: 6WX4).

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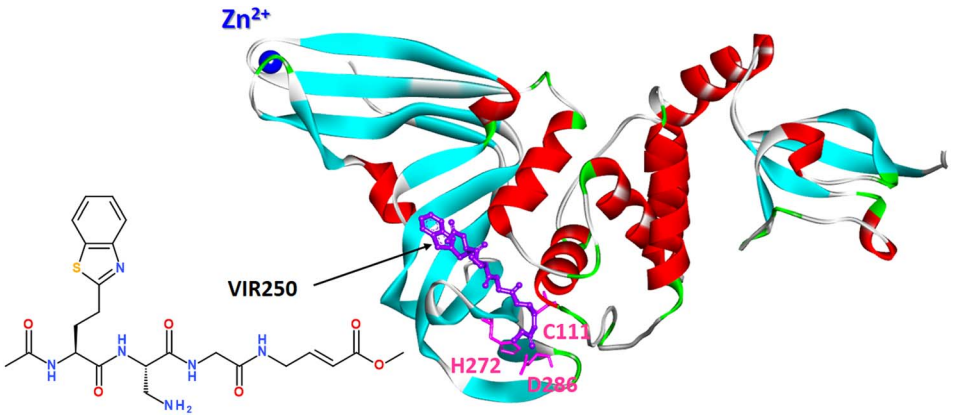

Fig. 20 X-ray structure of PLpro in complex with covalent peptide inhibitor VIR250 (PDB: 6WUU).

CoV-1 and SARS-CoV-2; however, they exhibited much weaker activity against MERS PLpro and human DUB UCH-L3.[152]

A molecular basis for the observed inhibitor specificity was provided to resolving X-ray structure of PLpro in complex with VIR250 peptide, as shown in Fig. 20.

The significant efforts are, however, focused on developing non-covalent inhibitors of PLpro.[149] Naphthalene-based non-covalent inhibitor GRL0617 (Fig. 21) was initially developed as an inhibitor of PLpro of SARS-CoV, and it did not show inhibitory activity against other host proteases.[160] Earlier trials revealed that the half-maximal inhibitory concentration (IC_{50}) of GRL0617 was 1.5 μM, so that its scaffold became a promising target for developing a series of its analogs.[147–149,161,162]

Recent study on hepatitis C virus (HCV) drug repurposing, has demonstrated that four HCV protease inhibitors, such as simeprevir, vaniprevir, paritaprevir, and grazoprevir, inhibited the SARS-CoV-2 PLpro too. So, HCV drugs that acting against PLpro synergized with the viral polymerase inhibitor remdesivir (Fig. 7) to inhibit virus replication, increasing remdesivir's antiviral activity as much as 10-fold.[163]

It has been shown that some human ubiquitin carboxyl-terminal hydrolase-2 (USP2) inhibitors, such as thiopurine analogs, were capable of inhibiting PLpro proteases. For instance, 6-thioguanine blocks SARS-CoV-2 replication by inhibition of PLpro with an EC_{50} of approximately 2 mM.[164] Using virtual screening combined with *in vitro* cytotoxicity studies, it was found that 1,3-benzothiazole derivative, known as ZINC9325709 or Z93, revealed the high potency against PLpro.[165]

In silico screening of a chemical libraries composed of >50k compounds allowed to design and synthesize other structurally diverse lead structures capable of noncovalent broad-spectrum inhibiting of coronavirus PLpro with low *in vitro* IC_{50},[166–168] as summarized in Fig. 22.

Beside the synthetic small-molecule inhibitors, some natural compounds, such as flavones, have revealed the inhibitory potency against PLpro enzyme.[101,105,169]

7.4. Dual and multi-targeting inhibitors of SARS-CoV-2 enzymes

Recent trends in the drug design against COVID-19 are focused on developing multi-target inhibitors for fighting several crucial proteins of SARS-CoV-2 with high potency.[167,170–174] The ability to identify inhibitors with dual inhibition against both Mpro and PLpro will increase the chances of success in the development of drug molecules against new variants of SARS-CoV-2.[53,175–178] Some studies have already reported compounds identified as

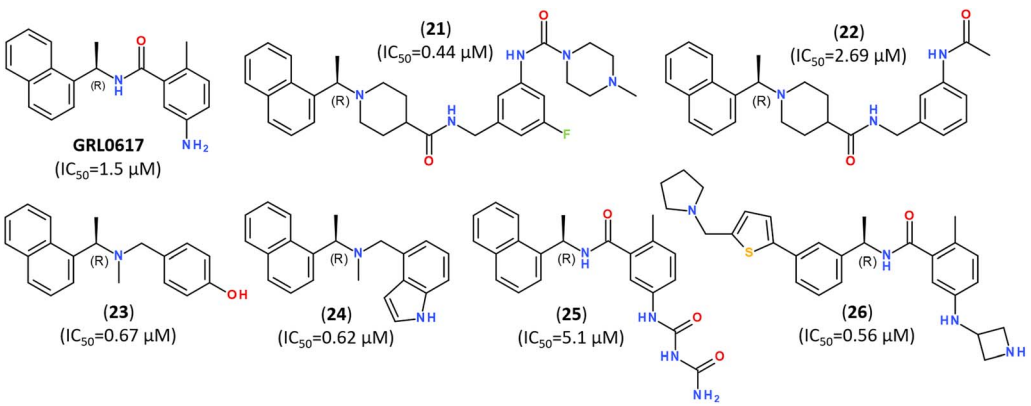

Fig. 21 Highly potent inhibitors of PLpro designed on a scaffold of the naphthalene-based inhibitor GRL-0617.

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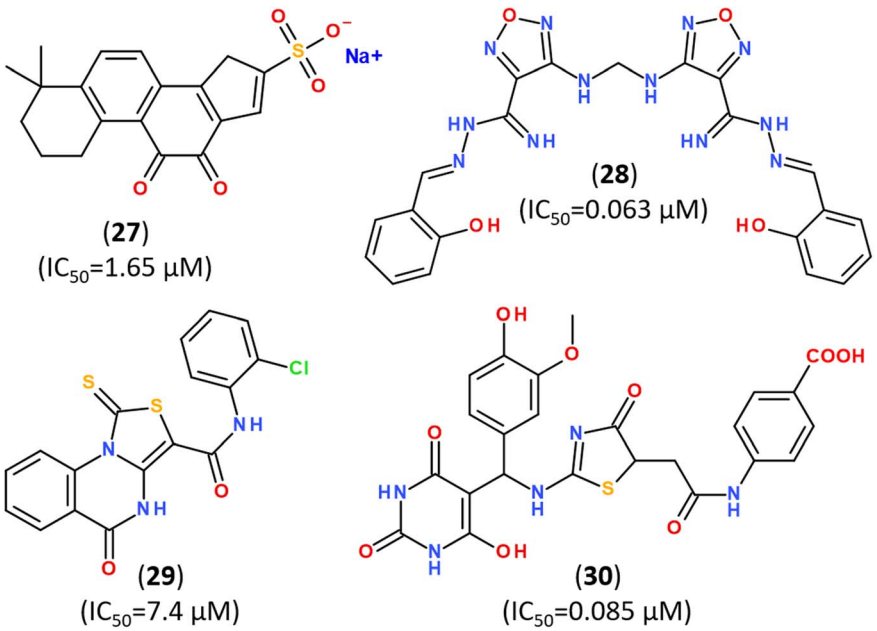

Fig. 22 Structurally diverse inhibitors of PLpro enzyme of SARS-CoV-2.

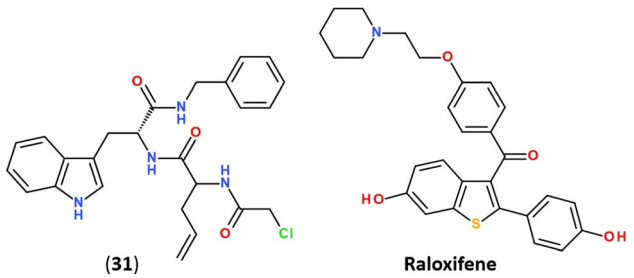

Fig. 23 Dual-targeting inhibitors against Mpro and PLpro enzymes.[132,177,180]

dual-acting SARS-CoV-2 protease inhibitors that target both Mpro and PLpro (Fig. 23).[179,180]

In silico screening of 688 naphthoquinoidal derivatives against Mpro protease of SARS-CoV-2 allowed to identify twenty-four derivatives for further biochemical assay evaluation, among which four hits were active against both Mpro and PLpro. These four hits inhibited Mpro with IC_{50} values between 0.41 μM and 9.0 μM, whereas, three of them inhibited PLpro with IC_{50} ranging from 1.9 μM to 3.3 μM, respectively.[179]

Some drug-like furo[2,3-*d*]pyrimidines and other 74 drug-like compounds were screened as potential active site inhibitors and putative allosteric hotspots modulators of SARS-CoV-2 Mpro and PLpro.[181] It has also been reported that some pertinent medicinal plants *i.e. Eurycoma harmandiana*, *Sophora flavescens* and *Andrographis paniculata* contain phyto-compounds, such as terpenoids, flavonoids and their glycosides, act against SARS-COV-2 PLpro and Mpro proteases.[80,173,182]

A combined approach for *in silico* screening towards repositioning of FDA-approved drug candidates for anticoronaviral therapy has identified Ouabain, which is an antiarrhythmic drug of plant origin, as a dual inhibitor for both PLpro and Mpro enzymes.[183]

Using *in vitro* studies and *in silico* screening, it has been shown that some purine nucleosides inhibiting nucleoside phosphorylase enzymes, such as Forodesine and Riboprine, revealed strong repurposing potential against two broadly conserved SARS-CoV-2 enzymes, such as RNA-dependent RNA polymerase (RdRp) and 3′-to-5′ exoribonuclease (ExoN), irrespective of the SARS-CoV-2 variant type (Fig. 24).[184,185]

Moreover, a newly discovered orally active noncovalent nonpeptidic agent Ensitrelvir (S-217622) (Fig. 13) has also revealed a synergistic triple noncovalent inhibitory activity against the three principal SARS-CoV-2 enzymes, namely: Mpro, RdRp, and ExoN by strong and stabilizing striking the key

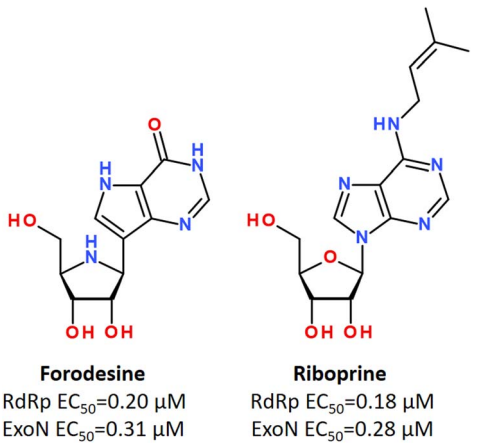

Fig. 24 Forodesine and Riboprine as dual acting inhibitors against RdRp and ExoN enzymes of SARS-CoV-2.[184]

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catalytic pockets of the SARS-CoV-2 RdRp's and ExoN's principal active sites.[114]

Finally, a recent *in silico* molecular docking study identified a FDA-approved antiviral agent, Rilpivirine, as a multi-target drug with high inhibitory potency against the Spike Glycoprotein and ACE2 receptors, PLpro and Mpro proteases, as well as RdRp protein, suggesting it may play a critical role in inhibiting the viral replication and assembly of the virus SARS-CoV-2.[55]

8. Summary and perspectives

The development of antiviral drugs for the treatment of COVID-19 continues to be relevant because of the risk of the appearance of SARS-CoV-2 mutated strains, reducing the effectiveness of existing vaccines. This review provides an overview of recent efforts in researching and developing coronavirus-related therapeutic agents. It includes an overview of coronavirus morphology, life cycle, and key proteins, focusing on antiviral strategies involving small molecule covalent and non-covalent inhibitors. The functional importance of Mpro and PLpro in the viral life cycle and the lack of close homologs in humans identify these proteases as an ideal target for screening of new inhibitors for SARS-CoV-2 replication. Active sites of Mpro are highly conservative for all coronaviruses. The review summarizes the drug-repurposing effort, focused primarily on inhibitors currently known to be effective against key cysteine-like proteases. In particular, Mpro and PLpro proteases of SARS-COV-2 were overviewed as therapeutic targets and their functional significance in the life cycle of the coronavirus was justified. Moreover, this report summarizes critical small molecule compounds discovered during the last three years, suitable for inhibiting several key coronavirus proteins.

The review outlines long-term drug development goals with a particular focus on a design strategy for dual-acting inhibitors that can inhibit both PLpro and Mpro proteases. It is believed that the combined therapy against both Mpro and PLpro inhibitors allows one to prevent the problem of drug resistance due to mutations in the viral genome.

Looking at the current scenario for the discovery of dual-targeting inhibitors, we can conclude that comparing pharmacophore models of Mpro and PLpro proteases may allow the identification of a set of spatial and electronic features, which are crucial for their interaction with biological targets. The combined pharmacophore model should include the pharmacophore features of both Mpro and PLpro models. The use of various selection criteria (stopping rule) makes it possible to determine the points of the best and worst regression model to identify an array of optimal options for the pharmacophore model for dual-acting inhibitors.

Author contributions

Larysa V. Yevsieieva: conceptualization, methodology, writing – original draft. Kateryna O. Lohachova: methodology, writing – original draft, resources. Alexander Kyrychenko: conceptualization, methodology, writing – review & editing. Sergiy M. Kovalenko: conceptualization, methodology, writing – original draft Volodymyr V. Ivanov: conceptualization, methodology, writing – original draft. Oleg N. Kalugin: writing – review & editing, supervision.

Conflicts of interest

There are no conflicts to declare.

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