



Antiviral peptides against SARS-CoV-2: therapeutic targets, mechanistic antiviral activity, and efficient delivery

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Abstract

The global pandemic of COVID-19 is a serious public health concern. Over 625 million confirmed cases and more than 6 million deaths have been recorded worldwide. Although several vaccines and antiviral medications have been developed, their efficacy is limited by the emerging new SARS-CoV-2 strains. Peptide-based therapeutics is a fast-growing class of new drugs and have unique advantages over large proteins and small molecules. Antiviral peptides (AVPs) are short polycationic antivirals with broad-spectrum effects, which have been shown to exert both prophylactic and therapeutic actions against reported coronaviruses. The potential therapeutic targets of AVPs are located either on the virus (e.g., E-protein and S-protein) to prohibit viral binding or host cells, particularly, those present on the cell surface (e.g., ACE2 and TMPRSS2). Despite AVPs having promising antiviral effects, their efficacy is limited by low bioavailability. Thus, nanoformulation is a prerequisite for prolonged bioavailability and efficient delivery. This review aimed to present an insight into the therapeutic AVP targets on both virus and host cells by discussing their antiviral activities and associated molecular mechanisms. Besides, it described the technique for discovering and developing possible AVPs based on their targets, as well as the significance of using nanotechnology for their efficient delivery against SARS-CoV-2.

Keywords SARS-CoV-2 · COVID-19 · Peptidase therapeutics · Antiviral peptides · Therapeutic targets · Efficient delivery · Nanoformulation

Abbreviations

AMPs	Antimicrobial peptides
ARDS	Acute respiratory distress syndrome
AVPs	Antiviral peptides
BSL	Bulged stem loop
CDC	Centre for disease control

COVID-19	Coronavirus disease 2019
EUA	Emergency Use Authorization
FP	Fusion peptide
HIV	Human immunodeficiency virus
HVR	Hyper-variable region
MERS	Middle Eastern respiratory syndrome

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MPs	Microparticles
NPs	Nanoparticles
NSPs	Non-structural proteins
PLA	Poly(lactic acid)
PLGA	Poly(lactic- <i>co</i> -glycolic acid)
RBD	Receptor binding domain
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TM	Transmembrane
US-FDA	United States Food and Drug Agency
UTR	Untranslated region

Introduction

Over the last 2 decades, there have been numerous epidemics, including severe acute respiratory syndrome (SARS) coronavirus, H1N1 influenza virus, Middle East respiratory syndrome (MERS) coronavirus, Ebola virus, and Zika virus [1–5]. Among these epidemics, the current global coronavirus disease 2019 (COVID-19) is the most contagious. The pathogen behind the COVID-19 epidemic is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [6]. An excess of 625 million individuals have been affected by the current pandemic due to SARS-CoV-2, with more than 6 million (approximately 1.04%) deaths recorded as of October 2022 [7]. Since its identification, the world scientific community has continually searched for treatments against SARS-CoV-2. Traditional approaches for drug discovery and development are time-consuming and can take an average of 15 years due to regulatory standards to analyze drug safety and efficacy. Currently, the US Food and Drug Administration (US-FDA) has approved Veklury, also known as remdesivir, an anti-viral drug for certain adult and pediatric cases. Additionally, the US-FDA has also approved monoclonal antibodies under the category of Emergency Use Authorization (EUA) for prophylactic and therapeutic use against COVID-19 [8].

Several vaccines against COVID-19 have recently been administered to human subjects under emergency use globally. According to the World Health Organization (WHO), approximately 60% of the world population has been fully vaccinated as of October 2022 [7]. Currently, three types of vaccines are employed to prevent SARS-CoV-2 infections, including messenger ribonucleic acid (mRNA), adenovirus vector-based, and inactivated virus-based vaccines [9]. Current US-FDA and WHO approved vaccines are produced by Pfizer-BioNTech (USA), Moderna (USA), Johnson & Johnson (USA), AstraZeneca and Oxford University (USA and UK), Sinopharm (China), Sinovac (China), CanSino Biologics (China), and Gamaleya (Russia), Novavax COVID-19 Vaccine, Adjuvanted

(USA), Serum Institute of India (India), Bharat Biotech International (India) [10–13]. Despite the rapid production and dissemination of vaccines, multiple new variants, such as alpha, beta, gamma, delta, and omicron, have emerged from various regions in the world, constituting the requirement for booster shots for enhanced prophylactic efficacy [14]. Other therapeutic approaches, such as the use of antiviral drugs, have been investigated for the treatment of COVID-19 patients. Remdesivir, hydroxychloroquine, and lopinavir-ritonavir are the three major antiviral drugs that have been utilized to treat COVID-19 [12]. However, hydroxychloroquine and lopinavir-ritonavir are not used as often because of their uncertain effectiveness [14]. Recently, the EUA of molnupiravir (Merck) as a new oral antiviral has been considered by the FDA [15]. On the other hand, the FDA has approved the EUA of paxlovid (Pfizer) as an oral antiviral treatment for patients who are at a high risk of developing severe COVID-19 [15]. Paxlovid is an inhibitor of SARS-CoV-2 3CL protease, while molnupiravir acts as a nucleoside analogue that leads to lethal mutagenesis of the viral genome [15].

Apart from vaccines and antiviral drugs, antiviral peptides (AVPs) have demonstrated preventive benefits against coronaviruses [16]. Antiviral polypeptides have been reported to have a significantly broad range of mechanism of action. For example, mucroporin-M1 induced destruction of the viral envelope [17], whereas targeting the heptad repeat 2 (HR2)-M2, EK1, EK1C4, and TMPRSS2 within the viral Spike (S) protein to inhibit protein-mediated fusion, the S2 subunit HR1 domain, and S-protein priming processes, respectively [18–20]. Other antiviral polypeptides prevent viral infection via other processes, such as lectin-like human defensins-5 (HD5) which binds and shields the host angiotensin converting enzyme-2 (ACE2) receptor [21], P9 which inhibits the late endosomal acidification to prevent viral RNA release [22], and RTD-1 which activates host-protective immune responses [23]. Thus, highlighting the heterogeneity of potential targets for novel therapeutics against SARS-CoV-2. Despite these benefits, antiviral peptides are particularly sensitive to breakdown by proteases and peptidases, leading to a short half-life and poor oral absorption. There is also the concern of delivery systems due to poor targeting and off-target toxicity. Taken together, these issues are required to be addressed by developing effective antiviral therapeutics with active targeting and low toxicity.

This paper presents a detailed review of antiviral activities and associated molecular mechanisms of different AVPs against SARS-CoV-2 according to their therapeutic targets. Furthermore, it discusses the strategy of identifying the potential targets of AVPs and the methods involved in the development of AVPs. Lastly, it presents the importance of using nanotechnology to deliver antiviral peptides against SARS-CoV-2 infection.

SARS-CoV-2

Origin, genome, and structure

SARS-CoV-2 is the causative agent of the ongoing global COVID-19 pandemic that infected 480 million individuals by April 2022 and caused over 6 million deaths [7]. The outbreak was first reported in China, where the source of infection of several patients with pneumonia of unknown origin was all linked to a Wuhan seafood market [24]. It was first identified as the novel coronavirus (2019-nCoV), and the name of the virus and disease were later changed to SARS-CoV-2 and COVID-19, respectively [25]. The virus falls under the genus betacoronavirus and family Coronaviridae, and it has a positive sense, single-stranded RNA genome of approximately 29.9 kb. The nucleotide sequence of SARS-CoV-2 shows 79 and 50% sequence homology with SARS-CoV and MERS, respectively [26]. Specifically, the SARS-CoV-2 and SARS-CoV S1 subunit of the S protein share approximately 50 conserved amino acids that otherwise showed mutational differences among other coronaviruses. Additionally, despite being categorized into the same phylogenetic clades as bat-derived coronaviruses, the RBD of SARS-CoV-2 shared higher genetic similarity with SARS-CoV, both of which bind to ACE2 receptor on the host cell membrane for viral entry. These findings suggested that there might be a similarity between the immune responses generated by these three coronaviruses [26].

The SARS-CoV-2 encodes 16 non-structural proteins (NSP-1–16), four structural proteins, such as the S, membrane (M), envelope (E), and nucleocapsid (N) proteins, and accessory proteins (Fig. 1). The 5' untranslated region (UTR) of the SARS-CoV-2 genome consists of two open-reading frames (ORFs) (i.e., ORF1a and ORF1b), which make up two-thirds of the viral genome, giving rise to the large polypeptides 1a (pp1a), and 1ab (pp1ab). These polypeptides are then cleaved into a total of 16 NSPs, where NSPs 1–11 are encoded by pp1a and pp1ab gives rise to NSPs 12–16 [27]. The functional involvement of 16 NSPs includes viral replication and transcription [28, 29]. The 3' end of the viral genome consists of conserved regions, such as the stem-loop-II-like motif (s2), hyper-variable region (HVR), mutually exclusive bulged stem-loop (BSL), which are involved in the synthesis of viral RNA [30].

The structural S protein is approximately 180–200 kDa in size and is made up of an extracellular N-terminal domain, receptor-binding domain (RBD) in the S1 subunit, an intracellularly located C-terminal domain, transmembrane (TM) domain, and heptad repeat 1 (HR1) and HR2 located in the S2 subunit [20]. The S protein is responsible

for host cell attachment and membrane fusion [16]. While the M protein is a transmembrane protein and comprises a long internal cytoplasmic domain, which creates an ion channel in the viral membrane and is also known as COOH terminus [25, 31]. While the E protein is an 8–12 kDa protein that is made up of an amino N terminus, a transmembrane domain, and a C terminus. It is involved in the virion assembly and release [26]. Lastly, the N nucleoprotein contains an N-terminal domain, central linker, dimerization domain, and C-terminal domain. This protein binds viral genome RNA to form a ribonucleoside-protein couple and is involved in viral RNA replication [27].

Eleven accessory proteins in SARS-CoV-2 have been reported: ORF3a, ORF3b, ORF3c, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10 (Fig. 1) [32]. The primary functions of the SARS-CoV-2 accessory proteins effect virulence and include improved viral entry into host cells, transport into cellular organelles, such as the nucleus for viral genome replication, evasion of host immune responses, and progression of cytokine storm [32, 33]. Among them, ORF3b, ORF6, ORF7a, and ORF8 accessory proteins are important type 1 interferon antagonists and have been associated with hijacking host-immune responses. ORF3a is known to induce apoptosis, while ORF9b and ORF9c are involved in suppressing host cell immune responses by interacting with cellular organelles. However, the biological roles of ORF3d, ORF7b, and ORF10 are still unknown [32].

Pathogenesis of SARS-CoV-2

Entry of SARS-CoV-2 into host cells is mediated by the S protein. The S1 subunit consists of the RBD and an N-terminal domain. The RBD binds specifically to the ACE2 receptor, indicating that SARS-CoV-2 RBD plays an important role in facilitating viral attachment to target host cells displaying ACE2 receptors [28]. Prior to membrane fusion, S protein exists in a prefusion native state and is made up of S1 and S2 subunits. However, once the RBD-ACE2 binding takes place, the S-protein undergoes conformational changes allowing proteolytic activation of the S1 subunit to be initiated, giving rise to the second conformation of the S protein, also known as pre-hairpin intermediate state. The S2 subunit is a class I fusion protein that is made up of a hydrophobic fusion peptide (FP), a long linking region, a transmembrane domain, and two HR1 and HR2. The anti-parallel conformation of the central HR1 helical coil and surrounding HR2 helices provides the structure with high stability, allowing it to overcome the energy barrier associated with membrane fusion [29, 34].

For membrane fusion to occur, the S protein is required to undergo a priming process, during which it is cleaved by host cellular serine proteases, particularly TMPRSS2,

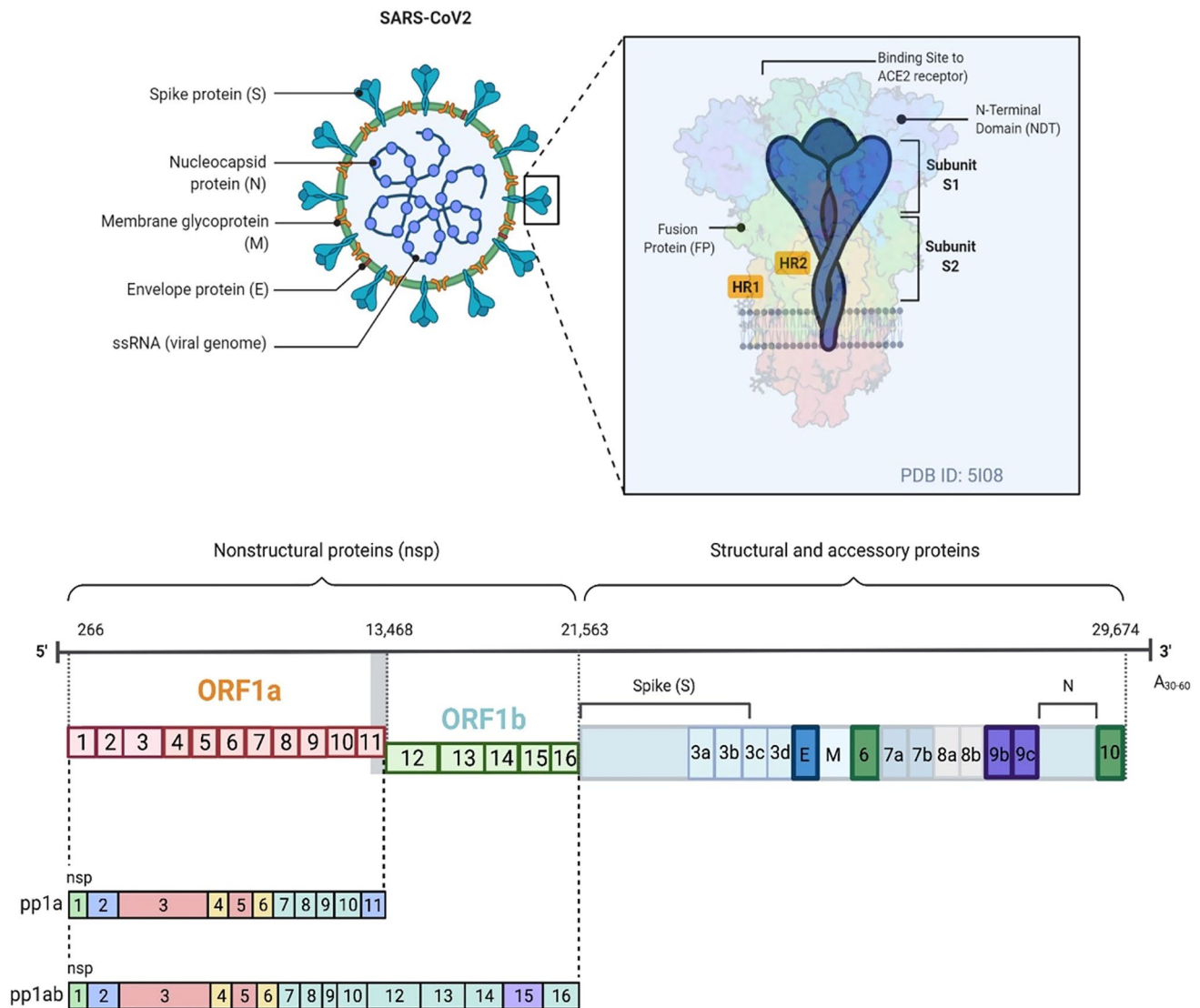


Fig. 1 The genetic structure and function of SARS-CoV-2. SARS-CoV-2 is made up of structural proteins, such as the envelope (E), spike (S), and membrane (M) proteins, as well as a nucleocapsid (N)

containing the viral RNA genome. The structural and accessory proteins are essential for SARS-CoV-2 pathogenicity

which is found on the host cell surface. This priming stage is essential for the viral capsid to be released into host cells, as it exposes hydrophobic FP on the S2 subunit, which is then inserted into the host cell membrane. Following insertion, HR1 and HR2 form a six- α -helix bundle (6HB) fusion core, also known as the stable post-fusion hairpin state, which brings the viral and host cell lipid bilayers into close proximity to begin the process of membrane fusion [30]. Following membrane fusion, SARS-CoV-2 enters host cells by forming endosomes. Viral RNA is then released into the host cell cytoplasm, where ORF1a and ORF1b undergo the process of translation, encoding for viral replicase polyproteins (e.g., pp1a and pp1b). These polyproteins are post-translationally modified by proteinases that are also encoded by the viral

genome, giving rise to NSPs that allow for viral replication and the formation of the transcription complex. Viral RNA and protein interaction occurring in the endoplasmic reticulum and Golgi apparatus result in the assembly of virions, which are then released from the host cell by exocytosis. The entry, replication, and viral assembly of SARS-CoV-2 in the host cells is presented in Fig. 2.

Immune response of host cells toward SARS-CoV-2 infection

Amid the progress of the infection, host cells protect themselves from destruction by triggering immune responses against SARS-CoV-2, particularly, preventing their

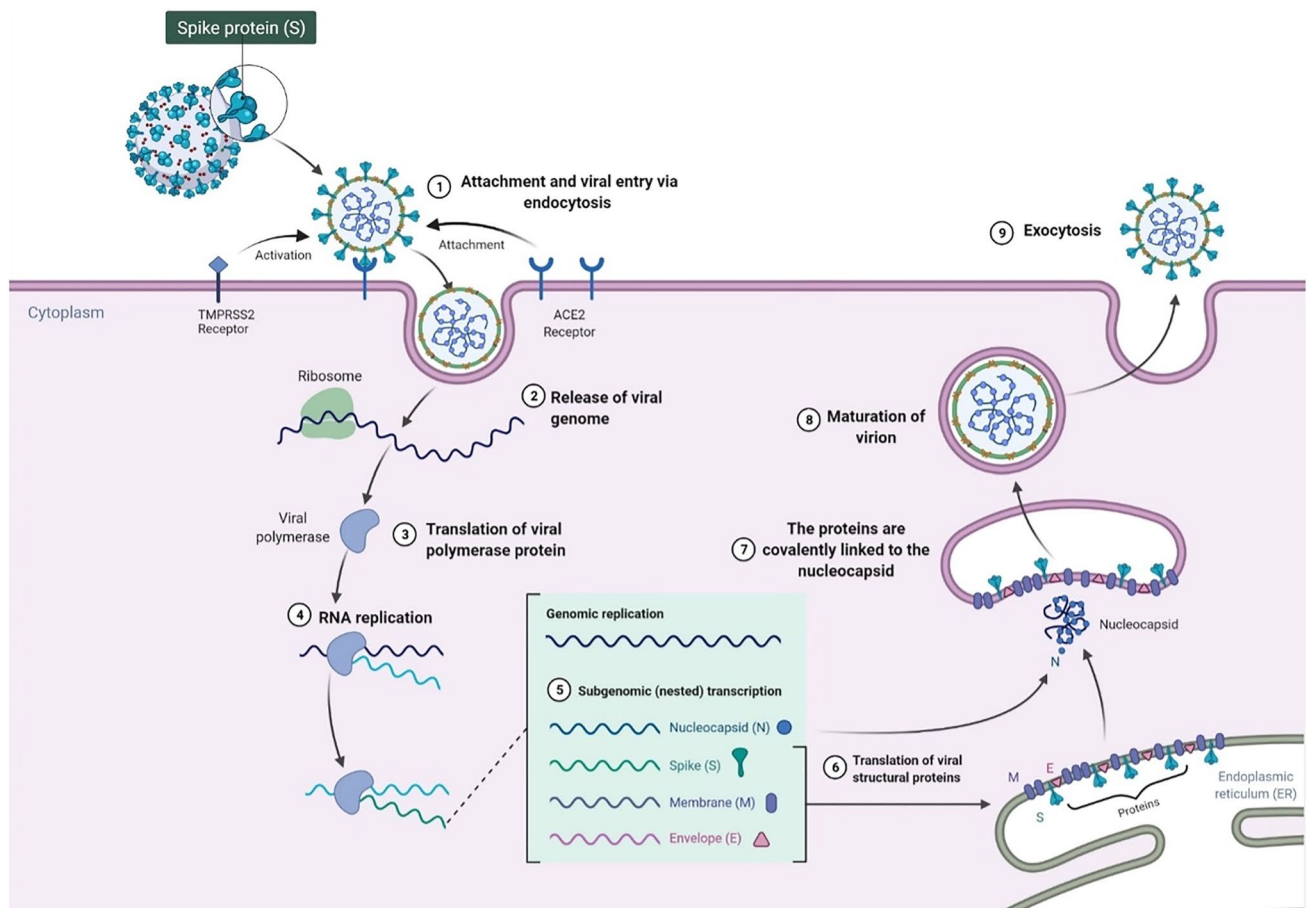


Fig. 2 Pathogenesis of SARS-CoV-2 to host cells. Attachment of SARS-CoV-2 RBS to the host cell membrane (ACE2) receptor (A) followed by membrane fusion, which is mediated by HR1 and HR2 domains on the S2 subunit of the S-protein. Formation of 6-HB

allows the virus to come into close proximity to the host cell membrane for membrane fusion to occur, followed by endocytosis, translation, replication, and maturation of the virus before exocytosis

replication and spread. Early innate immune defense mechanisms that have been identified as playing a role in protection against the spread of SARS-CoV-2 include the production of defense proteins and phagocytosis of the virus itself or infected host cells by mononuclear phagocytic cells, such as macrophages. This process is initiated by the recognition of pathogen-associated molecular patterns and has been reported to give rise to a “cytokine storm” [35, 36]. Among the proinflammatory cytokines that have been associated with COVID-19, IL-1, TNF- α , and IL-6 have been strongly linked to the inflammatory responses and may be directly linked to the unfavorable prognosis associated with severe COVID-19 [37–39].

Alternatively, adaptive immune responses are triggered if the innate immune responses are insufficient to prevent the replication of viral RNA and the synthesis of viral particles. Both humoral and cellular responses against SARS-CoV-2 have been reported. The humoral responses include the production of anti-SARS-CoV-2 RBD IgG antibodies with

neutralizing activity [40, 41], as well as increased antibody-secreting cells during the acute phase of COVID-19 [42]. Similarly, To et al. reported 100 and 94% seropositivity rates for anti-RBD IgG and IgM, respectively, identifying strong humoral responses against the RBD of the SARS-CoV-2 S-protein [43]. Additionally, the production of antibodies by the B-cell-mediated humoral immune responses has also been shown to cause an increase in SARS-CoV-2-specific IgG levels, particularly during the early stages of infection. Furthermore, both IgG and IgM possess neutralizing activity [44]. On the other hand, T-cell-mediated cellular responses have been observed in patients with asymptomatic to mildly symptomatic COVID-19, during which neutralizing antibody levels are lower than severe infection. Cellular responses that have been characterized during acute infection include increased SARS-CoV-2N and S protein-specific CD4+ T-cells [45]. Additionally, another study reported that patients recovered from mild COVID-19 showed 100 and 70% S-protein-specific CD4+ and CD8+ T-cell responses,

respectively [46]. The major differences in the immune responses during severe infection have been noted [47], including a lowered number of CD4+ and CD8+ T-cells, in patients requiring intensive care. Furthermore, disease progression to severe stages caused a significant increase in the expression of T-cell immune inhibitory surface markers (e.g., Trim-3 and PD-1), indicating functional exhaustion [48].

In addition to their protective functions, both innate and adaptive immune responses generated during SARS-CoV-2 infection are also correlated with the pathological damage to the host. One of the most significant immunopathologies involved in SARS-CoV-2 infection is cytokine storm, which has been identified as being a major cause of death in COVID-19 patients [16]. The release of inflammatory mediators, such as nitric oxide and reactive oxygen species, have been linked to respiratory distress as they can cause increased vascular endothelial permeability, leading to the damaged alveolar epithelium and impaired gas exchange due to extravasation of inflammatory cells [49, 50].

Clinical manifestations of SARS-CoV-2

SARS-CoV-2 infection appears to be transmissible to people of all ages, and the clinical manifestation also differs with age. The severity of infection varies, especially for people over 65 or people with comorbidities, including cardiovascular disease, hypertension, diabetes, and renal failure who are at greater risk of developing severe pneumonia to acute respiratory distress syndrome (ARDS), respiratory failure, mucormycosis, and multiple organ failure, that can lead to death. While infected children and young adults are commonly asymptomatic, recent studies have shown that children and newborns have a lower morbidity rate than adults. Therefore, COVID-19 patients may have a wide range of clinical manifestations based on their age and severity of infection [51–53]. The dominant clinical manifestations of SARS-CoV-2 include fever, cough, and shortness of breath. Subsequent clinical manifestations such as chills, muscle pain, headache, sore throat, diarrhea, skin rash, or discoloration of fingers or toes, red or irritated eyes, and loss of taste or smell were included by the US Center for Disease Control and Prevention (CDC) [53, 54].

Some patients show early symptoms like rhinorrhea, nausea, chest tightness, loss of speech or mobility, or confusion and vomiting before the onset of fever, suggesting that fever is not only considered as an early symptom but also a critical condition. The incubation period of SARS-CoV-2 is between 1 and 14 days, and in most cases, it is about three to seven days. Most of the time, the infected patients showed symptoms of infection after the incubation period, while shortness of breath and pneumonia developed within an average time of eight days [54, 55]. On the contrary, some patients only

show mild fever, mild fatigue, or even asymptomatic. This group of patients generally recover after one week, while severe cases have been documented that may develop progressive respiratory failure, as a result of alveolar destruction which can cause death [56].

Viral target identification and validation of antiviral peptides

AVPs are peptides that can inhibit viral infection or replication. The mechanism of action for each AVP type is different based on its target. These sites of action or inhibition can be found on the virus or host cell and are involved in essential steps of the viral replication cycle. Increasing interest towards the use of AVPs against many viral infections, including SARS-CoV-2, has been generated during the pandemic. This phenomenon is primarily due to their high specificity and potent antiviral activity even at low doses. Furthermore, many AVPs can be derived from natural or biological sources and have fewer associated adverse effects and cytotoxicity [57]. Intriguingly, the FDA has approved protease inhibitors, including both peptides (e.g., Enfuvirtide) and peptidomimetics mimicking the structural and functional properties of peptides (e.g., Boceprevir, and Telaprevir) for use against infectious diseases due to their ease of synthesis, greater efficacy, safety, and tolerability [58, 59].

Protein–protein interactions are essential for many cellular processes, including viral infections, and have been identified as central targets for drug discovery and development [60]. For instance, therapeutic peptides are target-specific peptides that are composed of less than 100 amino acids and are highly specific [61]. Furthermore, they are also non-toxic, because they can easily be hydrolyzed by normal host peptidases, thus preventing accumulation in organs that can lead to toxicity and other adverse effects. Some essential properties for AVPs include hydrophobicity, which is considered essential for targeting enveloped viruses specifically, as well as high target specificity, which is required for all types of AVPs to be effective [62].

Due to the complete dependence of viruses on the host cell machinery to complete most of the stages in their replication cycle, AVPs can be developed by targeting either the virus or host cells. Stages in viral replication that can be targeted include viral attachment, entry, uncoating, synthesis, and assembly [63]. The mechanism of action used by AVPs is generally summarized as being virucidal, as they are specifically designed or chosen based on their ability to interfere with essential steps in the viral life cycle to prevent viruses from establishing successful infection within the host cells. AVPs can be designed to interact with the viral membrane or structural proteins to successfully prevent viral attachment or entry. On the other hand, developing host-directed antiviral

peptides is a promising alternative strategy with a reduced likelihood of resistance [64].

Several strategies are currently used in the development of AVPs, including computationally derived peptides, peptides derived from plants, animals, and microorganisms, or peptides isolated from biological sources [59, 63]. Computational methods of AVP identification and design include molecular docking and simulations, as well as peptidomimetics [63, 65]. The process of molecular docking, where the interactions between molecules are analyzed by computational modeling focusing on their structural orientations and conformations, has accelerated drug development. This method provides the means to identify potential target sites and peptides with the highest binding affinity via an *in silico* approach. While peptidomimetics is another approach in which the biological target is mimicked, and its interaction with other proteins is examined. Both *in silico* and computational methods allow early identification of potential targets for AVPs. However, AVPs identified based on these methods are required to be validated using *in vitro* and *in vivo* approaches to gain a deeper understanding of their biological activities and antiviral efficacies.

The second approach that has been used in AVP development is identifying natural compounds showing potential antiviral activity [63]. The natural compounds are usually antimicrobial peptides (AMPs) that may have potent antiviral activity and can be isolated from natural sources, such as plants, animals, fungi, and bacteria. Depending on the mechanism of action, the properties of these natural AMPs and AVPs allow them to target viral proteins that are prerequisites for viral replication. One example of a naturally occurring peptide family with a broad-spectrum antiviral activity is cyclotides. These plant-derived AVPs have cationic and amphipathic properties, and they have been reported to prevent dengue, human immunodeficiency virus (HIV), and influenza infections by causing rupture of the viral membrane [66–68]. Comparatively, AMPs are often preferred over synthetic antiviral drugs due to their higher resistance to mutations and show a broad spectrum of antimicrobial activity [69].

Besides, the biological approach using *in vitro* display methodologies can also be used to identify and generate potential AVPs. They are usually genetically encoded to generate peptides with higher binding affinities to their targets. The most common techniques used in the biological approach include peptides displayed by phage, mRNA, ribosome, and yeast peptide libraries [59]. The phage display method fuses exogenous peptide sequences into the genome of a phage for surface expression, whereas mRNA display method extracts transcription and translation machinery from prokaryotic or eukaryotic cells to perform *in vitro* translation of covalently bonded mRNA polypeptide complexes linked through puromycin. The ribosome display

method uses *in vitro* translation of non-covalent-ribosome-mRNA-polypeptide complexes to couple genotypes and phenotypes which can select high-affinity peptides. Lastly, the yeast display method relies on the integration of the protein or peptide of interest on the cell surface glycoproteins via N- or C-terminal fusion. Yeast proteins that are used include both Ag- α -1 and aga-2, due to their role in yeast cell mating. The biological approach is considered the most appropriate method of screening different peptides for their potential antiviral activity, as the high affinity for their targets is determined using *in vitro* techniques.

SARS-CoV-2-specific targets for AVPs

As mentioned earlier, AVPs can be designed to target proteins that play an essential role in the viral replication cycle. The potential therapeutic targets of AVPs against SARS-CoV-2 can be categorized based on their location in either the viral cells or host cells, particularly, on the cell surface. The AVP viral targets are those that are involved in the prevention of viral infections by inhibiting viral entry into the host cells, including viral E-protein and S-protein sites (e.g., RBD and HR1/HR2 domains), as well as the viral membrane. Viral glycoproteins have been identified as preferred target sites for antiviral drugs in the past due to their importance in viral attachment and entry [70]. In agreement with this, two major sites that have been identified as potential targets in the AVP development against SARS-CoV-2 are the S1 and S2 subunits of S protein, which can prevent the virus from binding to host cells [71]. These targets are more favorable due to their extracellular location as they induce a lower risk of adverse toxicity to host cells. AVP host cell targets, on the other hand, are those that are involved in facilitating and allowing the viral S protein binding and entry (e.g., ACE2 and TMPRSS2). The following sections describe the therapeutic targets of AVPs on both viral and host cells, as well as their mechanistic activities, both of which have been summarized in Table 1.

Targeting the SARS-CoV-2 S-protein

SARS-CoV-2 S protein. S protein is a major site of interest in the identification and development of binding and/or attachment-inhibiting AVPs that have broad-spectrum activity against SARS-CoV-2 and other coronaviruses. This is because S proteins of viruses in the Coronaviridae family have similar nucleotide sequences. Particularly, the RBD of SARS-CoV and SARS-CoV-2 share 73–76% similarity in their nucleotide sequences [32]. Jaiswal and Kumar [72] used *in silico* methods to design an AVP targeting the S protein of SARS-CoV-2. The AVP of interest, Δ ABP-D25Y, was designed based on the ACE2 α -helical region [72]. Using molecular docking simulations, they found that

Table 1 Potential preventive and therapeutic targets for antiviral peptides against SARS-CoV-2

Peptide	Sequence	Viral/host target	Targeted virus	Method	Mechanism of action
ΔABP-D25Y [66]	not determined	Viral	SARS-CoV-2	Molecular docking and dynamic simulation	Inhibits RBD from binding to ACE2 receptors
S2P25 [67]	FGGASCC LYCRCHID HPNPKGFC DLGKY	Viral	Molecular docking and dynamic simulation	Inhibits RBD from binding to ACE2 receptors	
S2P26 [67]	ALNCYWPL NDYGFYTT TGIGYQPYR VVVLSFEL	Viral	SARS-CoV-2	Molecular docking and dynamic simulation	Inhibits RBD from binding to ACE2 receptors
EK1 [22]	SLDQINVTF LDLEYEMK KLEEAIAKKL EESYIDLKEL	Viral	SARS-CoV-2, SARS-CoV, and MERS,	In vitro detection of SARS-CoV-2 inhibition and in vivo mouse infection study	Prevents viral entry into host cell by competitively binding to HR1 domain
EK1C4 [22]	SLDQINVTF LDLEYEMK KLEEAIAKKL EESYIDLKEL GSGSG-PEG4-Chol	Viral	SARS-CoV-2, SARS-CoV, and MERS	In vitro detection of SARS-CoV-2 inhibition and in vivo mouse infection study	Prevents viral entry into host cell by competitively binding to HR1 domain
HR2-based antiviral peptide [74]	1168-DISGINASV-VNIQKEIDRL-NEVAKNLNES-LIDLQEL-1203	Viral	SARS-CoV-2	Molecular dynamic simulation	Inhibit viral entry by blocking fusion core formation
Mucroporin-M1 [75]	LFRLIKSLIK RLVSAFK	Viral	Measles, SARS-CoV, and influenza H5N1	In vitro measurements of antiviral effects	–
13a [80]	Not determined	Viral	SARS-CoV-2	Peptidomimetics, in vitro and in vivo measurements of antiviral effects	Inhibit viral RNA replication
P9 [81]	NGAICWGPCPTAFRQI-GNCGHFKVRCCCKIR	Viral	Influenza A virus H1N1, H3N2, H5N1, H7N7, H7N9, SARS-CoV and MERS-CoV	In vitro and in vivo measurements of antiviral effects	Bind to viral surface to prevent endosomal acidification, resultant basic endosomal pH to inhibits viral uncoating and release of viral genome
P9R [91]	NGAICWGPCPTAFRQI-GNCGRFRVRCCIR	Virus	SARS-CoV-2	In vitro measurements of antiviral effects	Inhibit endosomal acidification to block release of viral genome
HD5 [68]	ATCYCRTGRCAT RESLSGVCEISGRLYR-LCCR	Host	SARS-CoV-2	Molecular dynamic simulation	Inhibit viral attachment

Table 1 (continued)

Peptide	Sequence	Viral/host target	target	Targeted virus	Method	Mechanism of action
α 1-antitrypsin [86]	Not determined	Host	TMPRSS2	SARS-CoV-2	In vitro measurements of antiviral effects	Inhibit protease activity of TMPRSS2 and blocking membrane fusion
MI-432 and MI-1900 [87]	Not determined	Host	TMPRSS2	SARS-CoV-2	In vitro measurements of antiviral effects	Inhibit protease activity of TMPRSS2 and blocking membrane fusion
MI-432 and MI-1900 in combination with MI-1851 [87]	Not determined	Host	TMPRSS2 and furin	SARS-CoV-2	In vitro measurements of antiviral effects	Inhibit protease activity of TMPRSS2 and blocking membrane fusion
– [92]	Not determined	Host	TMPRSS2 and CatB/L	SARS-CoV-2	Mathematical modelling	Inhibit both membrane fusion and release of viral genome

the synthetically designed AVP might have the ability to inhibit SARS-CoV-2 infection by competitively blocking RBD interaction with ACE2 receptors on host cells. Additionally, Chowdhury et al. [73] also used molecular docking to screen 51 AVPs with known antiviral activity against SARS-CoV for their ability to bind to SARS-CoV-2. They found that S2P25, S2P26, and 13 other peptides that have higher binding affinity for the α -helical region of the RBD showed the most promising SARS-CoV-2-specific antiviral activity [73]. Contradictory to the appeal of using the same AVP against SARS-CoV and SARS-CoV-2 due to the similarity in their S protein, both SARS-CoV specific murine monoclonal antibodies and polyclonal antibodies were found to lack the ability to interact with SARS-CoV-2 RBD [73].

The S2 subunit of SARS-CoV and SARS-CoV-2 have approximately 90% nucleotide sequence identity, which is higher than the RBD in the S1 subunit, hence, it is a more desirable target site for AVPs with broad-spectrum anti-coronavirus activity [74]. Due to the critical role of HR1 and HR2 of the S2 subunit in membrane fusion, they have been identified as potential targets in AVP development [32]. In fact, AVPs that target these fusion sites have the potential to confer broad-spectrum antiviral activity against coronaviruses. One such fusion inhibitor is EK1 which has pan-coronavirus antiviral activity and is effective against SARS-CoV, SARS-CoV-2, and MERS-CoV infection [32, 75]. EK1 interferes with the viral infection by binding to and blocking the HR1 domain, therefore inhibiting viral entry into the host cells and preventing the formation of downstream 6HB core. Furthermore, EK1 has shown both prophylactic and therapeutic effects, making it more desirable than peptides that confer either effect. Based on previous knowledge, the conjugation of cholesterol to existing AVPs has resulted in improved antiviral activities, as documented in the use of C34 peptide against HIV-1 [76]. Similarly, Xia et al. [20] conjugated a cholesterol-moiety to the C-terminal of EK1 using a glycine/serine linker and PEG spacer. The resulting lipopeptide, known as EK1C4, showed 226-fold higher binding affinity for the HR1 domain and about 240-fold higher antiviral activity against SARS-CoV-2 than EK1 alone [77].

AVPs designed to target the H2 domains of enveloped viruses have potent antiviral activity. The first AVP to be approved for clinical use is a fusion blocking peptide that successfully inhibited HIV infection by binding to the HR2 domain of the HIV envelope virus [76, 77]. Additionally, anti-MERS-CoV HR2P-M2 peptide also has demonstrated antiviral activity by targeting the H2 domain [78]. Recently, Ling et al. [79] used in silico approaches to design a HR2 targeting AVP against SARS-CoV-2. They hypothesized that the binding of the synthetic AVP to the HR2 domain might prevent the formation of pre-hairpin conformation, which consequently inhibited SARS-CoV-2 infection [79]. The results showed that using AVPs that bind specifically

to HR2, the binding of HR1 and HR2 was blocked. As a result, the fusion core that is essential for membrane fusion was not formed. In silico design was based on identifying and modeling both HR2 domain and fusion core, as well as the entire S protein before molecular docking. In addition to its extracellular target site and having both prophylactic and therapeutic effects, AVPs targeting the HR1 domain are superior to AVPs targeting the HR2 domain as the sequence of the HR-1 is relatively highly conserved. Besides, peptides that are modeled for binding to the HR1 domain are also predicted to have higher resistance against mutations that may lead to antiviral resistant viral strains [63].

Targeting the SARS-CoV-2 E protein

SARS-CoV-2 E protein. Another target of AVPs is the viral E protein. One such AVP that has shown potential antiviral activity against a broad-spectrum of enveloped viruses (e.g., SARS-CoV, MERS-CoV, influenza H5N1, and measles) is mucroporin-M1 [70]. This cationic AMP, an analog of mucroporin, is a toxin isolated from *Lychas mucronatus* venom, and it contains four mutations at positions of G3R, P6K, G10K, and G11R. When enveloped viruses, for instance, coronaviruses attach to the host cells, a series of morphological changes take place that subsequently increases the binding affinity of mucroporin-M1 (molecular blocker) to the viral E protein, followed by viral envelope disruption. Additionally, the peptide variant was also reported to have antibacterial properties, making it an attractive therapeutic against bacterial and viral co-infections, such as secondary bacterial infections seen in severe SARS-CoV-2-induced pneumonia [80]. However, as compared to the S protein, the E protein is not a major site of interest. This is because it has previously been demonstrated it has a low copy number and the presence of conflicting evidence on its importance in viral replication [81].

Targeting SARS-CoV-2 non-structural proteins

SARS-CoV-2 non-structural proteins. SARS-CoV-2 main protease (Mpro) is a NSP and has been previously identified as a potential target for antiviral drugs designed against SARS-CoV-2 [82]. The protease enzyme carries out the processing and digestion of polyproteins that are encoded by viral RNA and controls the cleavage of the overlapping pp1a and pp1ab into 1-NSPs. Additionally, it is also responsible for the release of other proteolytic enzymes involved in viral replication, such as NSP 13, making it essential for viral maturation [82]. SARS-CoV-2 Mpro is a homodimer that is consisted of two protomers and three domains, with domains I and II being composed of six antiparallel β -barrels, while domain III is made of five α helices. The proteolytic activity of Mpro is conferred by N-terminus and Cys-His catalytic

diad located in the cleft between domains I and II. It is conserved across all coronaviruses, making it an ideal target for antiviral drugs with broad-spectrum activity against all human coronaviruses [83, 84].

Zhang et al. used in silico methods to predict the antiviral activity of SARS-CoV-2 Mpro inhibiting α -ketoamide, which is also known as 13a [83]. The design of the compound was based on modifying 11r, which was previously identified as having broad-spectrum activity against coronaviruses and adenoviruses by specifically targeting main proteases of both alpha and beta coronaviruses and 3C protease of enteroviruses. To design 13a, 11r was modified by hiding the P3-P2 amide bond in a pyridine ring, followed by replacing hydrophobic cinnamoyl moiety with a relatively less hydrophobic Boc group to prevent cellular proteases from cleaving the amide bond and increasing solubility in plasma for a decreased plasma protein binding, respectively. Using peptidomimetics, it was shown that 13a potentially had SARS-CoV-2 specific antiviral activity by binding with the SARS-CoV-2 Mpro to prevent viral maturation by inhibiting RNA replication.

One other approach that has been utilized to confer a broad-spectrum antiviral activity is the use of peptides to interfere with the viral uncoating stage, which in turn inhibited the release of genetic material into the host cells. For instance, P9 peptide, derived from mouse beta-defensin-4, could bind to SARS-CoV, MERS-CoV S-proteins, and multiple influenza viruses (e.g., H1N1, H3N2, H5N1, H7N7, and H7N9) [85]. However, rather than blocking the binding sites needed for cell–cell interaction, P9 remained attached to the viral surface as it entered the host cell. Once inside the endosome, the polycationic properties of P9 resulted in a basic microenvironment rather than the acidic microenvironment, which is vital for these viruses to release their genetic material. The basic pH prevented viral membrane fusion with the host endosomal membrane, thus preventing viral RNA release.

Antiviral peptides targeting host cell targets

ACE2 receptor. Rather than targeting structures on the viral surface, the ACE2 receptor of host cells also can be targeted, as this would also prevent viral entry. An AVP targeting the host cell receptor required for viral binding and entry has already been approved for treating HIV-1 [86]. Maraviroc, an HIV-1 entry and fusion inhibiting AVP, was able to prevent HIV-1 entry into host cells by selectively binding to human chemokine receptor CCR5, which directly interferes with the crucial step of HIV-1 gp 120 binding to CCR5. Similarly, a hexapeptide Tyr-Lys-Tyr-Arg-Tyr-Leu designed from a naturally occurring hexapeptide found in the RBD of SARS-CoV was shown to reduce viral infection in vitro using epithelial cell lines [87]. Based on the results, this

AVP bound to host cell receptors and interfered with necessary interactions for viral attachment. However, the activity of this hexapeptide against SARS-CoV-2 has not yet been examined. Next, HD5 peptide is a peptide secreted by Paneth cells in the crypts of Lieberkühn that has a high affinity toward ACE2 receptors [74]. The molecular dynamic simulation was used to show that HD5 is competitively bound to ligand-binding domains on the ACE2 receptor. Additionally, it was found that HD5 and ACE2 binding occurred through the formation of multiple hydrogen bonds to confer protection against SARS-CoV-2 that would otherwise bind via the free ACE2 receptor.

Host cell proteases. Due to the crucial role of proteolytic cleavage in host cell entry, TMPRSS2 also has been targeted and shown to effectively inhibit SARS-CoV-2 entry into host cells [28]. An abundant naturally occurring serine protease inhibitor, α 1-antitrypsin, was shown to prevent SARS-CoV-2 entry into host cells by inhibiting the protease activity of TMPRSS2, leaving the S2 domain uncleaved and therefore unable to undergo membrane fusion [88]. Other than SARS-CoV-2, TMPRSS2 is also an important host cell protease in the replication of influenza A [89]. The role of TMPRSS2 in several viral infections would allow AVPs that target the host cell protease to have broad-spectrum activity against respiratory viruses. Bestle et al. demonstrated the role of peptide mimetic inhibitors of TMPRSS2 (MI-432 and MI-1900), that could successfully inhibit SARS-CoV-2 from causing infection in human airway cells [90]. Interestingly, the antiviral activity was improved when both TMPRSS2 inhibitors were combined with the furin inhibitor MI-1851, indicating potential synergistic effects of peptides that target different cleavage sites.

Another host protease that can be targeted is CatB/L, which is a lysosomal cysteine protease that plays a role in SARS-CoV-2 host cell entry via the endosomal pathway [28, 91]. CatB/L is required for S1 subunit cleavage prior to fusion of host cell endosomal and viral membrane. Therefore, the release of viral genetic material can be inhibited by blocking CatB/L [92]. Other than designing peptides that competitively bind to the CatB/L protease, peptides that inhibit endosomal acidification can also be used to inhibit the activity of CatB/L and other host cell proteases that is pH-dependent [93, 94]. Zhao et al. (2020) reported the potent broad-spectrum activity of P9R against enveloped viruses. The mechanism of action used by this AVP is the inhibition of endosomal acidification by reducing protons within the endosome [95]. The results showed that P9R, which was modified to have a more positive charge, had higher SARS-CoV-2 specific antiviral activity than the previously tested P9, which was also designed to bind to viral glycoproteins and prevent endosomal acidification [85, 95]. In addition to inhibiting either TMPRSS2 or CatB/L using antiviral peptides, Padmanabhan et al. (2020) showed better

antiviral activity and complete inhibition of viral entry when both proteases were targeted simultaneously [96]. Targeting both TMPRSS2 and CatB/L have been suggested to be a more effective method in preventing viral infection, as it inhibits both routes of entry that can be used by SARS-CoV-2 to enter host cells. This is particularly more advantageous than targeting a single route of entry, as the virus particles can enter host cells via another route.

Efficient delivery of potential SARS-COV-2 using nanoformulations

It has been reported that the use of AVPs as therapeutics is limited due to their instability, short half-life, and easy degradability, all of which lead to poor bioavailability. On top of that, they are also associated with having low potency and poor ability to cross membrane barriers [79]. Particularly, naturally derived AVPs are unstable and have demonstrated weak binding affinity to their targets, sensitive to conformational changes in response to environmental stimuli, limiting the ability to cross membranes due to large size, and poorly excreted [97]. Thus, these physicochemical properties may limit their clinical application. Besides, AVPs have limited systemic delivery that may prevent them from reaching the target site with the correct dose due to the presence of numerous proteases, as well as rapid clearance from blood circulation by opsonization and agglutination [96].

These limitations can be resolved using nanoformulations (Fig. 3). For instance, nanoparticles (NPs) are known for having physical properties that can be manipulated, making them optimized drug carriers. Advantages of using NPs include protecting peptides from proteolytic and enzymatic degradation, prolonged bioavailability via sustained peptide release, and preventing peptides from premature clearance, all of which extend their bioavailability. Furthermore, NP encapsulation can be used to enhance the delivery of hydrophobic or insoluble AVPs. Systemic side effects induced by peptide-based therapeutics also can be reduced using NP encapsulation, as it can ensure targeted drugs to release in a controlled and consistent manner. Examples of nanocarriers that can be used include polymeric NPs, such as poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), chitosan, liposomes, and micelles [98]. These NPs can be selected or modified to ensure they are non-toxic, non-immunogenic, biodegradable, and suitable for peptide and protein encapsulation. On top of that, their size, surface area, stimuli-responsiveness, and surface charge also can be modified to enhance their delivery to specific target sites and further improve the efficacy of antiviral peptides [99]. Among different types of NP-encapsulated AVPs, polymeric peptides have demonstrated antiviral activity against influenza, herpes simplex virus, human papillomavirus, respiratory syncytial virus, dengue, and lentivirus [100, 101].

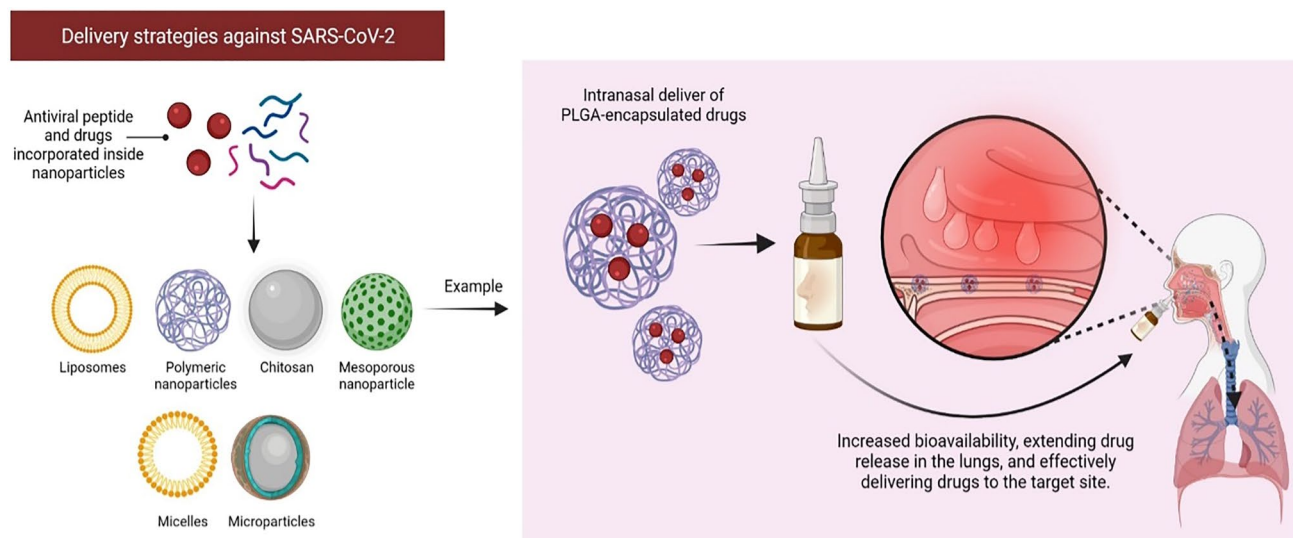


Fig. 3 SARS-CoV-2 virus transmission mechanism. SARS-CoV-2 is airborne, and once inhaled, the virus's spike proteins may have a strong affinity for the mucins in the mucus that lines the airways. Nanoparticles can be used to load drugs and AVPs against SARS-

CoV-2 due to their capacity to shield the cargo from proteolytic and enzymatic degradation, as well as prolonging and increasing its bio-availability

Using NPs as drug carriers also may potentially allow for non-conventional/needle-free administration, such as intranasal administration [99, 102]. The intranasal route is favorable for drug administration, as it eliminates the need for trained healthcare professionals to deliver the vaccine and reduces the cost of immunization programs, making it easily accessible to third-world countries and regions with low socioeconomic status [103]. Furthermore, ease of drug administration has been associated with increased patient compliance [104]. Additionally, the main route of entry used by SARS-CoV-2 includes the airways, from which they travel to the lungs prior to entering the systemic circulation. In this regard, AVPs against SARS-CoV-2 can be administered intranasally, as the nasal mucosa provides a large surface area for nanoparticle absorption for both local and systemic delivery of peptides [105]. Intranasal delivery of AVPs was shown to be effective against respiratory infections caused by MERS in animal models. The use of HR2P-M2, a MERS fusion inhibitor was shown to exhibit high efficacy as both a prophylactic and therapeutic [106]. It was also demonstrated that intranasal application of EK1C4 to mice could protect them from pre- and post-human coronavirus OC43 infections, indicating that it has both prophylactic and therapeutic effects against currently circulating SARS-CoV-2 and other emerging SARS-related coronaviruses [20]. Additionally, pulmonary delivery of AVPs may also be a preferred route of administration to target the localization of SARS-CoV-2 in the lungs, attributing to the large surface area and thin membrane barriers of the alveoli, which provided faster drug carrier absorption into the blood circulation [61]. Given

these advantages, polymeric NPs, specifically PLGA, have been preferably applied for pulmonary drug delivery due to their biodegradability, prolonged drug release within lungs, and slow degradation rates [61, 107]. Besides, the formation of nanocomposites with NPs encased in sugar microparticles (MPs) has been used to overcome the limitations associated with intranasal administration of PLGA-encapsulated drugs [108]. For SARS-CoV-2, the use of modified PLGA nanocarriers to deliver peptide-based antiviral drugs is considered highly preferred, as it can target SARS-CoV-2 both locally within the lungs and systematically in other organs through absorption into the blood circulation [109].

Conclusion

SARS-CoV-2 is the world's third most virulent human coronavirus and is highly contagious due to its rapid spread and life-threatening infections in the aged population. To date, the primary method for prevention of COVID-19 infection relies on vaccination, thus conferring the need for the identification of an effective oral antiviral for prophylactic use. Several antiviral strategies are continuously being investigated, but no specific antiviral treatment has been confirmed until recently [110, 111]. Furthermore, there is no guarantee that COVID-19 vaccinations will provide long-term effective protection, and this prevention approach is certainly inapplicable and ineffective for those who have been infected by SARS-CoV-2 variants of concern. Therefore, the use of AVPs for the treatment of newly developing viral infections

holds a lot of potentials due to their safety and effectiveness. Intriguingly, AVPs have been demonstrated to interact with therapeutic targets against coronaviruses, either on virus or host cells. The viral targets of AVPs are those present on the virus to prevent their binding and entry to host cells, including E protein and S protein sites, while AVP host cell targets are particularly those present on the host cell surface (e.g., ACE2 and TMPRSS2). Mechanistically, AVPs target viral membrane or structural proteins to inhibit them from infecting host cells successfully. Additionally, AVPs can also prevent SARS-CoV-2 entry into host cells, particularly by inhibiting the binding to host cell receptors or masking viral peptides that are essential for successful infection [63]. Comparatively, there is a relatively higher risk for viruses to develop resistance as compared to AVPs designed to target the host cell proteins [62] due to the fact that a high rate of mutations may occur in the viral genome [57]. It has been reported that therapeutics application of AVPs is limited due to their instability, short half-life, easy degradability, and poor bioavailability. To overcome these limitations, nanoformulation approaches have emerged as a promising biological strategy to improve their distribution and stability against SARS-CoV-2, particularly, using intranasal and pulmonary delivery, and loading into PLGA nanoparticles. NP-encapsulated AVPs have shown encouraging results for both preclinical and clinical applications [110].

Eleven known accessory proteins have been identified, but their biological and regulatory roles in SARS-CoV-2 pathogenesis are still largely unknown. Thus, it is necessary to develop AVPs targeting them and evaluate their associated anti-SARS CoV-2 activities and molecular mechanisms. Accumulating studies demonstrate that non-coding RNAs, particularly, long non-coding RNAs, are essential regulators of SARS-CoV-2 infection by affecting viral gene expression, replication, and pathology in the host cells, as well as to evade the immune response of host cells [108, 112–114]. Besides, further exploration of potential natural or biological sources such as cyclotides (plant-derived AVPs) which could exert broad-spectrum or unique promising antiviral effects with less associated adverse effects and toxicity should be conducted. The current studies investigating the potential application of NPs to deliver AVPs for treating COVID-19 are limited, thus more investigations for their preclinical efficacy and pharmacokinetic profiles should be performed to enable clinical translation. Interestingly, the application of NPs to deliver AVPs allows them to be structurally and functionally versatile, which could serve as the molecular template for the development of advanced therapeutic applications in the face of the current pandemic threat. Additionally, the potential application of aptamers, a type of bio-inspired receptor comprised of single-stranded DNA or RNA, should also be considered as an approach to deliver therapeutic AVPs or formulated as a SARS-CoV-2 targeting agent. For

instance, AVP-based aptamers can bind to identical amino acids of RBD, thus providing a promising biological tool for COVID-19 prevention, and treatment [115].

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Declarations

Conflict of interest The authors declare no conflict of interest.

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