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REVIEW

Chemically modified antiviral peptides against SARS-CoV-2

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To date, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) COVID-19 pandemic continues to be a potentially lethal disease. Although both vaccines and specific antiviral drugs have been approved, the search for more specific therapeutic approaches is still ongoing. The infection mechanism of SARS-CoV-2 consists of several stages, and each one can be selectively blocked to disrupt viral infection. Peptides are a promising class of antiviral compounds, which may be suitably modified to be more stable, more effective, and more selective towards a specific viral replication step. The latter two goals might be obtained by increasing the specificity and/or the affinity of the interaction with a specific target and often imply the stabilization of the secondary structure of the active peptide. This review is focused on modified antiviral peptides against SARS-CoV-2 acting at different stages of virus replication, including ACE2-RBD interaction, membrane fusion mechanism, and the proteolytic cleavage by different viral proteases. Therefore, the landscape presented herein provides a useful springboard for the design of new and powerful antiviral therapeutics.

KEYWORDS
antiviral peptides, chemical modifications, COVID-19, SARS-CoV-2

1 | INTRODUCTION

The *Severe Acute Respiratory Syndrome Coronavirus 2* (SARS-CoV-2), isolated in the city of Wuhan, China, in early 2020, continues to be a health threat. The infection, termed *Coronavirus Disease 19* (COVID-19), can occur through a wide range of symptoms, from a simple cold to severe respiratory crises that can be even fatal. Since the pandemic started there 761 million cases and 6.8 million deaths globally have been confirmed.[1]

Immunization through vaccines has drastically reduced severe cases of COVID-19 and remains the most effective way of prevention nowadays. On the other hand, this defense is linked to the activity of the vaccinated immune system, which in some cases may be hampered, as in immunocompromised individuals.[2,3] Therefore, it is necessary to develop antiviral compounds as therapeutics, in case the vaccine does not work in predisposed individuals. Two antiviral drugs are currently available to prevent the development of severe forms of the disease: Molnupiravir (Lagevrio®) that is a prodrug of the synthetic nucleoside derivative N^4-hydroxycytidine and exerts its antiviral action by introducing copying errors during viral RNA replication[4–6] and Nirmatrelvir (Paxlovid®) that is a peptidomimetic, which acts as an orally active SARS-CoV-2 protease inhibitor.[7–9] Although these compounds are widely used, the research for new, more selective, and effective antivirals is ongoing.

The mechanism of infection of SARS-CoV-2 is complex and consists of several steps: entry into the host cell, replication, and transcription and finally assembly and release (Figure 1). The first event regards the viral entry in which a key role is played by the Spike glycoprotein of SARS-CoV-2, a trimeric protein composed of the subunits S1 and S2.[11] The viral replication cycle is triggered through the interaction between a specific fragment contained in the S1 subunit of Spike, called *Receptor Binding Domain* (RBD), and the transmembrane

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FIGURE 1 Scheme of the SARS-CoV-2 lifecycle. The three steps in which modified peptides may act as inhibitors are highlighted. Modified from Brady et al.[10]

receptor *Angiotensin Converting Enzyme 2* (ACE2), present on the host cell surface.[12] This interaction induces a conformational change in the Spike glycoprotein that enables the proteolytic cleavage of its S1 subunit by the *Transmembrane protease serine 2* (TMPRSS2) of the host cell.[13] Then, the N-terminal fragment of S2, called the *Fusion Peptide* (FP), interacts with the host cell membrane triggering the interaction between Heptad repeat region 1 and 2 (HR2 and HR1). This intraprotein interaction leads to the formation of a six-helix bundle (6-HB), bringing the viral membrane closer to the cell membrane and allowing the virus to transfer the genetic material (RNA) inside the host cell (Figure 2).[15,16]

The second step involves the translation of the viral genome (ssRNA+), in particular the open reading frames (ORF1a/b) into two polyproteins pp1a and pp1ab. These are then cleaved by two cysteine proteases, such as papain-like protease (PL^{pro}) and 3-chymotrypsin-like protease ($3CL^{pro}$) also called major protease (M^{pro}), and transformed into nonstructural proteins (nsps). Subsequently, the viral replication and transcription complex (RTC) is formed, which includes several nsps, including the RNA-dependent RNA polymerase (RdRp), essential for viral RNA replication. In the cytosol, the new viral RNA is packed into a helical structure by the interaction with multiple copies of the *Nucleocapsid Protein*. Then, the viral envelope is formed, and finally, the virions are secreted by exocytosis.[17–19]

The complexity of the SARS-CoV-2 replication cycle requires the development of specific antiviral compounds. On the other hand, this complexity can be exploited to design therapeutics, which targets one or more phases in this cycle, thus blocking the infection. Among all the many compounds developed in this context, we focussed our attention on peptides suitably modified to increase specificity and antiviral activity against SARS-CoV-2. In fact, bioactive peptides are versatile drug candidates, since it is possible to combine the many desirable features of a native amino-acid sequence with the possibility to install a large variety of chemical modifications (thanks to the reactive functional groups) to improve its drug-like properties. Accordingly, we reviewed the large number of modified peptides endowed

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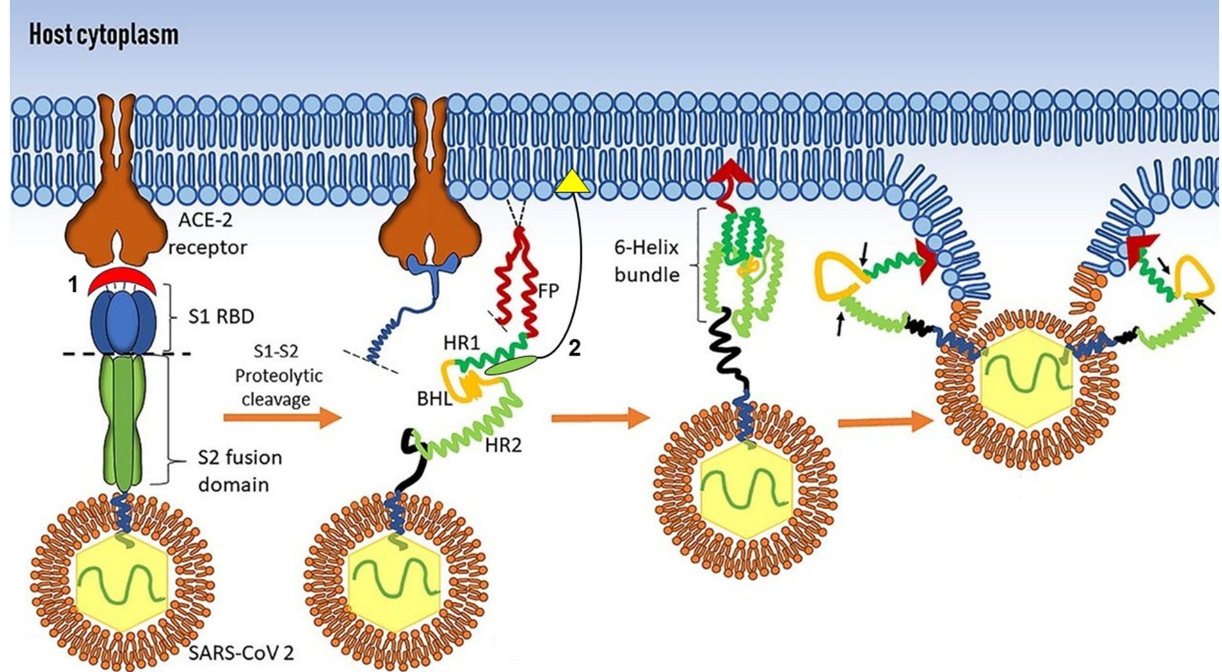

FIGURE 2 Site of action of peptide inhibitors. (1) ACE2 analogs (in red) bind to spike RBD and inhibit the interaction with ACE2 on the cell surface, thus blocking viral entry. (2) HR2 analogs (in green), possibly modified with a lipophilic tail (in yellow), interact with HR1 blocking the six-helix bundle formation. Modified from Shekhar et al.[14]

with anti-SARS-CoV-2 activity and described in the last 3 years. To emphasize the importance of chemical modifications of native amino-acid sequences, we excluded from this analysis those peptides that have been pharmacologically tested in their native, unmodified form.

Currently, almost 100 peptides are in active clinical development, with many more in preclinical studies.[20–22] The use of peptides as therapeutics has multiple advantages. For example, these molecules have high specificity, high efficacy, and are generally low in toxicity.[23] In addition, solid-phase peptide synthesis (SPPS) reduced the time and cost of production of peptides in the drug development phase, while robust hybrid methods were developed for their large-scale GMP-compliant production, as required for active pharmaceutical ingredients (APIs). On the other hand, native peptides have a low capacity to cross the cell membrane and are not very stable to hydrolytic enzymes in the biological system.[24] In addition, activity and selectivity of peptides also depend on the secondary structure, which generally differs from the isolated sequence and the same sequence inserted within the protein.[25] This is a relevant issue when the peptide is designed as an inhibitor of a particular protein–protein interaction (PPI). In light of these considerations, it is therefore necessary to modify native peptides to turn drawbacks into advantages.

Low proteolytic stability remains one of the most relevant disadvantages of native peptide sequences. However, there are modifications that enable to increase the half-life in biological systems. For example, the introduction of nonnatural amino acids that are not recognized by proteases can increase the stability.[26] Among these, the D-amino acids are widely used. In fact, it has been demonstrated that only a few enzymes can hydrolyze the amide bond involving D-amino acids.[27] In addition, D-residues can stabilize a particular conformation and thus influence selectivity for a specific target.[28,29] Other widely used nonnatural residues are β-amino acids and fluorinated amino acids, both inducing increased enzymatic stability.[30,31]

Another drawback to consider is the poor cell permeability of peptides, which does not allow the application of these molecules to intracellular targets. A methodology that is frequently used to facilitate the cell-entry is the addition of a particular amino acid sequences called cell penetrating peptides (CPPs).[32,33] Among these, the most widely used sequence is a nonamer of the transactivator of transcription (TAT) from the human immunodeficiency virus (HIV), termed TAT peptide.[34] The use of the TAT sequence is exploited not only to improve cell permeability of peptides but also for the internalization of nanocarriers, including gold nanoparticles.[35–37] Another widely used modification is the addition of a lipidic moiety.[38] In fact, lipid moieties increase the affinity to the cell membrane favoring insertion into the membrane, which then promotes cellular uptake.[39,40] Generally, long-chain fatty acid moieties, such as the palmitoyl one, are used as lipid tags because increasing the number of methylenes increases cellular uptake.[41–43]

Frequently, stabilization of secondary structures can strongly affect both activity and selectivity of bioactive peptides. However, under physiological conditions, the interactions that stabilize the secondary structure are weakened and the peptide tends to assume a random-coil conformation, reducing or losing its activity. Formation of intramolecular covalent bonds can reduce the degrees of freedom, rigidifying the peptide structure. This result can be achieved by

cyclization and can be performed exploiting the side chains or the peptide backbone. The head-to-tail cyclopeptides are widespread in nature and exhibit a variety of biological activities.[44,45] In addition, it was demonstrated that cyclopeptides have both higher proteolytic stability and enhanced cellular uptake than linear analogs.[46,47] On the other hand, side chain cyclization, also known as "stapling", can be formed in several ways.[48] In general, this strategy consists of replacing residues that are not important for activity with modified amino acids suitable for the bridge formation.[49]

In light of these considerations, modified peptides with antiviral activity against SARS-CoV-2 will be analyzed and classified according to the different viral target (highlighted in Figure 1), with the aim of providing a rational useful tool for the development of new and more potent compounds.

2 | MODIFIED PEPTIDES AS INHIBITORS OF ACE2-RBD INTERACTION

As mentioned above, the interaction between the Spike fragment RBD and the receptor ACE2 is the first event occurring between the virus and the host cell. In this scenario, a molecule capable of interfering with this interaction blocks the activation of the cell entry, as shown in Figure 2. According to the cryo-EM structure of the ACE2-Spike S1 complex (PDB: 6M0J), the ACE2 key residues involved in this interaction are S19, N24, T27, D30, K31, H34, E35, E37, D38, Y41, and Q42, located in the α1 helix and K353, G354, D355, and R357 that are located in the β sheets β3 and β4.[50,51] Thus, research efforts have been focused on the α1 fragment of ACE2, which contains most of the residues involved in the interaction. Nevertheless, native peptides based on the α1 sequence do not maintain a helical structure in solution and thereby display limited ability to efficiently bind to RBD.[52,53] Therefore, these peptides were modified to stabilize their secondary structure and thus increasing efficacy (Table 1).

There are several ways to stabilize the secondary structure, but the use of "stapled" to obtain conformationally constrained peptides is the most widespread. The simplest way to achieve this result is the formation of a lactam bridge via the side chains of Lys and Asp residues.[62] Mass et al. exploited this type of reaction to synthesize different ACE2(21–55) stapled analogs changing the lactam-bridges position.[54] It was demonstrated that shifting the lactam to the N-terminal increases the α-helix propensity. In particular, the stapled analog at position 36–40 was reported to have the most stable secondary structure and higher inhibitory activity in RBD-ACE interaction, with an $IC_{50} = 3.6$ μM assessed by ELISA (**Entry 1,** Table 1). Interestingly, the lactam bridge in positions 28–32 and 32–36 destabilizes the structure of the α-helix.

Regarding the α1 fragment of ACE2, other types of stapled have been studied, including *Ring-Closing Metathesis* (RCM), which allows to obtain highly hydrophobic alkenyl-bridges.[63] This reaction is versatile and is used to obtain longer bridges such as *i*, $i + 7$ by increasing the number of methylene groups in the alkenyl-terminal side chains.[64] Curreli et al. synthesized and measured the antiviral activity of di-stapled peptides derived from the ACE2 fragment (20–49) using RCM to stabilize the secondary structure.[55] The analog termed NYBSP-4 (**Entry 2,** Table 1), which contains 2 alkenyl bridges in positions 25–32 and 43–47, has 80% of α-helical propensity in PBS and inhibits the

TABLE 1 Modified peptides inhibitor of ACE2-RBD interaction.

Entry	Target	Name	Sequence	Ref.
1	RBD	hACE2$_{21\text{-}55}$A36K-F40E	IEEQAKTFLDKFNHE-NHCHCO-EDL-NHCHCO-YQSSLASWNYNTNIT	Maas et al.[54]
2	RBD	NYBSP-4	Ac-TIEEQ-NHCCO-KTFLDK-NHCCO-NHE-NHCCO-EDL-NHCCO-YQSSLASWN-NH_2	Curreli et al.[55]
3	RBD	Peptide 6	HE-NHCCO-EDL-NHCCO-YQ-NH_2	Calugi et al.[56]
4	RBD	P3	QAKTFLDKFNHE-NHCHCO-EDL-NHCHCO-YQ	Quagliata et al.[57]
5	RBD	P-2-2	Ac-βHAsp-PPEQAKTFLDKFNHEAEDLFYQK-NH_2	Engelhardt et al.[58]
6	RBD	P10	SALEEQYKTFLDKFMHELEDLLYQLAL-NH_2	Karoyan et al.[59]
7	RBD	P6cyc	EEQAKTFLDKFNHE-NHCCO-EDLFYQ-NHCCO-SGLGKGDFR	Sarto et al.[60]
8	RBD	CSNP2	LQAANFLDK-NHCHCO-NDE-NHCHCO-EDLFYKPGG-NHCHCO-LGKGD-NHCHCO-RIL	Shah et al.[61]

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infection in human fibrosarcoma HT1080/ACE2 cells and in the human lung carcinoma A549/ACE2 cells with $IC_{50} = 1.97 \pm 0.14$ and 2.86 ± 0.08 μM, respectively. In the same direction, Calugi et al. synthesized a shorter analog derived from the fragment ACE2(34–42) containing an alkenyl bridge at position 36–40 (**Entry 3,** Table 1).[56] This modified peptide is able to inhibit the RBD-ACE2 interaction with an $IC_{50} = 21 \pm 7$ μM assessed by ELISA. Interestingly, this stapled analog, in addition to having a more stable secondary structure than the native peptide, exhibits greater proteolytic stability in human plasma over 48 h.

Another more recent method used for the development of stapled peptides is the formation of a triazolyl bridge exploiting the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC).[65–68] We used this approach to perform a structure–activity relationship with mono- and ditriazolyl bridged peptides derived from the minimal sequence ACE2(24–42).[57] In particular, the analog clicked at position 36–40 features a more stable secondary structure and increased antiviral activity as compared to the native fragment (**Entry 4,** Table 1). Interestingly, the analog containing a double triazolyl-bridge results in the highest propensity of α-helix (96.2% in 1:1 TFE:H_2O), but this higher rigidity reduces the antiviral activity, demonstrating that the flexibility of the C-terminal part is also important.

Recently, it has been shown that N-capping of a peptide using the rigidified diproline-derived module termed *βHAsp*-ProM-5 increases the α helical content.[69] With this in mind, Engelhardt et al. synthesized some ACE2(24–42) minimal sequence analogs by adding this building block in N-terminal position (**Entry 5,** Table 1). The propensity of the α-helix increases dramatically compared with the analogs without the modification, but they show a modest affinity for the RBD fragment of Spike ($K_d = 1.21 \pm 0.36$ μM) in microscale thermophoresis (MST).[58] Interestingly, the N-capping with the nonnatural motif *βHAsp-Pro-Pro* enhances the affinity ($K_d = 0.062 \pm 0.017$ μM) without increasing the helical content. The use of nonnatural amino acids was then exploited by Karoyan et al. for the synthesis of an ACE2(19–45) fragment derivative in which several leucine residues were inserted to increase a-helix propensity and an L-homotyrosine (*h*Tyr) (**Entry 6,** Table 1).[59] In particular, a key role in the interaction seems to be played by this nonnatural residue, as the peptide named P10 has a much higher affinity for RBD ($K_d = 0.03 \pm 0.01$ nM) than the native fragment containing Ala, as assessed by biolayer interferometry.

The examples reported above involved the α1 portion of ACE2. As previously mentioned, there are other residues located on the β sheets β3 and β4 also important for the interaction with RBD. Moreover, these residues are located spatially close to the helix α1. Sarto et al. synthesized a conformational stapled chimera composed of the two key fragments in ACE2 to improve binding affinity.[60] The hydrocarbonyl-bridged compound called p6cyc (**Entry 7,** Table 1) exhibits an interesting dissociation constant ($K_d = 270 \pm 140$ nM) assessed by MST using RBD. In the same direction, Shah et al. proposed a similar di-stapled chimera, characterized by a lactam bridge in N-terminal and a disulfide bridge in C-terminal.[61] This peptide, called CSNP2 (**Entry 8,** Table 1), showed a high antiviral activity in vitro ($IC_{50} = 0.37$ μM) and a modest binding affinity for the subunit S1 ($K_d = 32.8$ μM) in the SPR assay.

3 | MODIFIED ANTI-FUSION PEPTIDES

Once binding between the RBD fragment of the viral Spike and the ACE2 receptor occurred, subsequent proteolytic cleavage of the subunit S1 leads to a series of conformational changes in the S2.[70] In particular, three HR1 domains form an internal trimer and interact with three HR2 to produce a six-helix bundle (6-HB), thereby bringing viral and cellular membranes in close proximity for fusion. In this scenario, peptides capable of interfering with the formation of 6-HB are termed antifusion peptides and are able to block SARS-CoV-2 cell entry, as shown in Figure 2. Generally, these potential therapeutics derive from sequences contained inside HR1 or HR2 which interact with the other domain (Table 2).

The formation of 6-HB requires the interaction of multiple domains characterized by an α-helix structure. As mentioned above, one of the most widespread ways of stabilizing the secondary structure is the use of staples that block the peptide conformation. An example of antifusion stapled peptide was reported by Zheng et al.,

TABLE 2 Modified antifusion peptides.

Entry	Target	Name	Sequence	Ref.
1	**HR1**	**SCH2-1-20**	Ac-DISGINASVVNIQKEIDLR-NHCCO-EVA-NHCCO-NLNESLIDLQEL-NH_2	Zheng et al.[71]
2	**HR1**	**EK1C16**	SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKELGSGSG-PEG_4-Pal	Xia et al.[72]
3	**HR1**	**EK1C4**	SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKELGSGSG-PEG_4-Chol	Xia et al.[73]
4	**HR1**	**P40-LP**	VDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL-PEG_8-K-Chol	Hu et al.[74]
5	**HR1**	**IPB02**	ISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELK-Chol	Zhu et al.[75]
6	**HR1**	**$[SARS_{HR2}$-$PEG_4]_2$-chol**	(DISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELGSGSGC-$PEG_4)_2$-Chol	Schmitz et al.[76]

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who described a peptide derived from Spike(1168–1203) entirely located in HR2.[71] The all-hydrocarbon stapled analog, bridged in position 1187–1191 and called SCH2-1-20 (**Entry 1,** Table 2), has a higher α-helix propensity than the native sequence and a stronger antiviral activity in vitro (55% of inhibition rate at 12.5 μM).

An important aspect to consider is that once proteolytic cleavage of the S1 subunit has occurred, the *Fusion protein* (FP) fragment of the S2 subunit interacts with the cell membrane bringing the HR1 fragment close to the host cell. This fact can be exploited to design more potent peptides by targeting the cell compartment where fusion occurs, through introduction of a membrane anchor in the form of a lipophilic group.[77–80] In this way, several HR2 analogs with different lipophilic linker were synthesized and tested. One of the most widely used is palmitic acid. In fact, palmitoylation of proteins is an important posttranslational modification (PTM), which is generated biologically by enzymes known as palmitoyl acyltransferases (PATs).[81] Moreover, several palmitic acid-based lipopeptide drugs were submitted to clinical trials.[82,83] In this scenario, Lan et al. synthesized a palmitoylated derivative of peptide EK1, a pan-CoV fusion inhibitor, called EK1C16 (**Entry 2,** Table 2).[72] In particular, the EK1 peptide was modified in C-terminal by adding a peptide spacer (GSGSG), a polyethylene glycol spacer (PEG_4), and finally, the palmitoyl group.[84] The results show that the lipopeptide EK1-C16 has a higher antiviral activity than EK1 in all the Variants of Concern (VOC) of SARS-CoV-2. For example, regarding the alpha variant, the inhibition is more than 10-fold stronger ($IC_{50} = 0.11$ vs. 1.21 μM).

Another approach exploits a specific feature of some regions of the cell membrane, called lipid rafts, characterized by accumulations of particular proteins and lipids. These tightly packed membrane microdomains are essential for the organization and assembly of signaling molecules, influencing membrane fluidity, and regulating the trafficking of membrane proteins, neurotransmitters, and receptors.[85–87] The abundant presence of cholesterol in lipid rafts suggests its use as an effective lipidic anchor.[88] To verify the key role of this sterol, Xia et al. synthesized the cholesteryl derivative of peptide EK1C16, termed EK1C4 (**Entry 3,** Table 2).[73] The presence of cholesterol instead of palmitic acid dramatically increases antiviral activity in vitro with an $IC_{50} = 15.8$ nm. Interestingly, intranasally applied EK1C4 showed strong protection of mice against HCoV-OC43 infection.

The key role of cholesterol in antifusion peptides is also reported in other studies. Hu et al. compared the activity of the native peptide Spike(1164–1203), entirely included in the HR2 region, with a C-terminal analog modified with a PEG_8 spacer and a cholesterol moiety, termed P40 and P40-LP, respectively (**Entry 4,** Table 2).[74] The antiviral activity of the cholesteryl-derivative is more than 1000-fold higher than the native sequence shifting from a mean $IC_{50} = 2.32$ μM to 1.99 nM, calculated against different SARS-CoV-2 variants. In the same direction, Zhu et al. designed the cholesteryl-derivative (without spacer) of the shorter fragment Spike(1169–1203), called IPB02 (**Entry 5,** Table 2). Here, again, the presence of the sterol in C-terminal dramatically increases antiviral activity in vitro, as compared with the native sequence ($IC_{50} = 33.7$ vs 0.08 μM).[75] Finally, Outlaw et al. demonstrated that the cholesteryl derivative of the fragment Spike(1168–1203) containing a C-terminal PEG_4 spacer is able to inhibits infectious SARS-CoV-2 viral spread in a human airway epithelial (HAE) in ex vivo model.[89]

As reported above, the formation of 6-HB involves the three helices of the HR1 and HR2 fragments. Therefore, increasing the number of copies of the antifusion peptides on the same molecule can increase its inhibitory ability. This fact was exploited by Schmitz et al., who synthesized a chimera, termed $[SARS_{HR2}\text{-}PEG_4]_2$-chol (**Entry 6,** Table 2), containing two HR2 peptides attached via a PEG_4 spacer to a cholesterol molecule.[76] The dimer was compared with the monomer, showing greater antiviral activity in vitro ($IC_{50} = 8$ vs. 2 nm), as assessed using the D614G variant of SARS-CoV-2. In the same paper, the authors also compared the activity of monomeric analogs with different lengths of the PEG spacer, finding no difference in the use of PEG_4 or PEG_{24}. Interestingly, the intranasal dimer-peptide chimera prevents SARS-CoV-2 transmission in ferrets, providing protection during a 24-h period of direct contact.[90]

4 | PROTEASE INHIBITOR PEPTIDES

Membrane fusion leads to virus entry into the cell. Infection then proceeds with translation and transcription of the viral genome leading to the formation of the polyproteins pp1a and pp1ab. These polyproteins are then cleaved by two types of proteases obtaining different non-structural proteins, which are essential for virion formation. Accordingly, molecules which can block the activity of these proteases have potent antiviral activity (Table 3).

4.1 | M^{pro} inhibitors

The main proteases (M^{pro}), also termed 3-chymotrypsin-like proteases ($3CL^{pro}$), are a class of highly conserved cysteine hydrolases. Proteolytic cleavage of M^{pro} generally occurs after a Gln residue, preceded by a hydrophobic residue, such as Leu, and followed by a small amino acid, such as Ala or Ser. Interestingly, there are no M^{pro} homolog proteases in human; therefore, inhibitors of M^{pro} are extremely selective and have reduced side effects due to inhibition of physiological proteases.[97] Generally, to block the activity of a protease, molecules which can occupy the active site in order to inhibit hydrolytic activity are used. This mechanism implies that such compounds have a relatively rigid and ordered structure. Regarding peptides, one way to stabilize the structure is to form macrocycles to reduce degrees of freedom.

It has been shown that the M^{pro} of SARS-CoV has a substrate specificity for its C-terminal autoprocessing. In fact, inactive forms of M^{pro} have a 10-amino acid C-terminal prosequence, which is cleaved by another M^{pro} at Gln306.[98] Therefore, based on previous studies performed on SARS-CoV and given the extreme similarity in both structure and sequence between M^{pro} of SARS-CoV and SARS-CoV-2, the prosequence can be exploited to design inhibitors, as it is recognized by the active site of the enzyme.[99] In this way, Kreutzer et al. synthesized a cyclopeptide derived from the sequence

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TABLE 3 Protease inhibitor peptides.

Entry	Target	Name	Sequence	Ref.
1	M^{pro}	UCI-1		Kreutzer et al.[91]
2	M^{pro}	Pen 1	O=C(NH-yLQYAVLRHKRRE-NHCHCO-RQIKIWFQNRRMKWKK-NH$_2$) bridged via S	Johansen-Leete et al.[92]
3	M^{pro}	MN-2	NH$_2$CHCO-FLQYAVLKQKRRE-NHCHCO-GGGRPPRRAR (S–S bridge)	Yin et al.[93]
4	M^{pro}	GM4H3Q	O=C(NH-yFQγNLNGYRPG-NHCHCONH$_2$) bridged via S	Miura et al.[94]
5	PL^{pro}	VIR251		Rut et al.[95]
6	PL^{pro}	Peptide 29		Di Sarno et al.[96]

M^{pro}(305–309).[91] In particular, their peptide, termed UCI-1 (**Entry 1,** Table 3), presents the mutation G307S and contains a [4-(2-aminoethyl)phenyl]acetic acid (AEPA) as a head to tail bridge to enforce a conformation that mimics a peptide substrate of M^{pro}. This modified peptide is able to inhibit M^{pro} activity with $IC_{50} = 160$ μM.

Regarding cyclic peptides, Johansen-Leete et al. identified several compounds using Random nonstandard Peptide Integrated Discovery (RaPID) technology, a biological method which allows the screening of $>10^{12}$ cyclic peptides for affinity against a protein target of interest immobilized on magnetic beads.[100] One of the most active products is the cyclopeptide 1, characterized by a head to tail thioether bridge formed by the side chain of Cys with N-terminal chloroacetic acid.[92] This modified sequence has a potent proteolytic activity inhibition with $IC_{50} = 0.070 \pm 0.018$ μM but has no antiviral activity due to its poor cell permeability. To increase cellular uptake, peptide 1 was further modified with penetrin, a CPP, resulting in compound pen-1 (**Entry 2,** Table 3), which showed promising antiviral activity in vitro ($EC_{50} = 15.9 \pm 0.7$ μM). In the same way, Yin et al. synthesized different disulfide cyclic peptides, selected by virtual screening using M^{pro} as a target.[93] Among these, the one called MN-2 (**Entry 3,** Table 3) has a strong affinity for M^{pro} ($K_d = 18.2 \pm 1.9$ nm) and also a promising antiviral activity in vitro against the variant Omicron BA.2.75 with about 80% inhibition rate at 2 μM. More recently, Miura et al. synthesized a library of cyclic peptides containing cyclic $\gamma^{2,4}$-amino acid (cγAA).[94] The analog termed GM4H3Q (**Entry 4,** Table 3), which has a head to tail thioether bridge and a *cis*-3-aminocyclobutane carboxylic acid residue, appears to be stable to human serum ($t_{1/2} = 82$ h) and also has potent inhibitory activity of M^{pro} ($IC_{50} = 10 \pm 0$ nm).

4.2 | PL^{pro} inhibitors

The papain-like protease (PL^{pro}) is another hydrolytic enzyme essential for SARS-CoV-2 viral replication. Differently from M^{pro}, the cleavage site of PL^{pro} is composed of the tetrapeptide LXGG motif and occurs on the carboxyl side of C-terminal glycine.[101] Peptide inhibitors of PL^{pro} are generally modified to bind covalently the Cys111, which belongs to the catalytic triad of the enzyme, together with His272 and Asp286.[102]

To date, there are two examples of covalent inhibitory peptides which exploit the "thiol-ene" click reaction to form an irreversible

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thioether with the side chain of Cys111.[103] The first, proposed by Rut et al., concerns a sequence which contains two nonnatural residues such as hTyr and 2,4-aiaminobutyric acid (Dab), and a C-terminal vinyl-methyl ester VME group (**Entry 5,** Table 3).[95] The VME motif plays a key role, as it is a powerful Michael acceptor and easily undergoes nucleophilic attack by thiol groups. The covalent interaction with Cys111 has been demonstrated by crystallography, and the modified peptide can inhibit the enzyme activity almost completely at 100 μM.

In the same direction, Di Sarno et al. synthesized a dipeptide composed of L-allylglycine and L-Trp, with the C-terminal aminobenzyl and the N-terminal capped with chloroacetic acid (**Entry 6,** Table 3).[96] This compound is able to inhibit the activity of PL^{pro} ($IC_{50} = 0.67 \pm 0.59$ μM) and has good antiviral activity against SARS-CoV-2 ($EC_{50} = 0.32$ μM).

5 | CONCLUSIONS

Despite the availability of vaccines, COVID-19 is still a life-threatening disease, especially for the more vulnerable subjects. Currently, two drugs against SARS-CoV-2 have been approved, but the complexity of the infection cycle requires the development of new therapeutics, more selective and effective. Peptides represent a category of compounds that address these requirements, but they have some disadvantages such as low stability in physiological conditions, poor cell permeability, and low conformational stability. These compounds, however, can be easily modified to obtain new peptides with unique characteristics, which can dramatically increase potency and selectivity. We reported in this review all the modified peptides active against SARS-CoV-2 described in the literature, classified according to the different targets, that is, the ACE2-RBD interaction, the membrane fusion process, and the two viral specific proteases, M^{pro} and PL^{pro}.

Among the latter, M^{pro} appears to be a promising target since a nonpeptide inhibitor has already been approved for the treatment of COVID-19. In fact, this protease lacks a human homolog, and therefore, its inhibition is potentially devoid of side effects due to inhibition of physiological proteases, a crucially positive factor for a drug. However, the intracellular localization of this target requires the development of cell permeable peptide analogs, a goal that can be reached, even if adding some complexity to the development of new drug candidates. On the other hand, fusion inhibitory peptides have been approved in the past for other viral diseases, such as AIDS, caused by human immunodeficiency viruses (HIV).[104] In this case, the target is extracellular, but it has been shown that interaction of the drug with the cell membrane through a lipid moiety, such as cholesterol, dramatically increases antiviral activity. Accordingly, cholesteryl antifusion peptides appear to be excellent candidates as potential drugs. Finally, from the synthetic viewpoint, the availability of new, more versatile and biocompatible cyclization methods, such as the formation of triazolyl bridges by click chemistry, will play a key role in the development of second-generation antiviral peptides. In conclusion, modified peptides appear to be useful weapons against SARS-CoV-2, and therefore, although none of them entered, up to now, the stage of clinical development, this review aims to provide a tool which can help the development of new, more potent and selective antiviral peptide analogs.

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