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HIGHLIGHT

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Stapled peptides as potential inhibitors of SARS-CoV-2 binding to the hACE2 receptor

Susan Tzotzos

Apeptico GmbH, Vienna, Austria

Correspondence
Susan Tzotzos, Apeptico GmbH, Mariahilfer Strasse 136, Vienna 1150, Austria.
Email: stzotzos@me.com

Stapled peptides are synthetic peptidomimetics of bioactive sites in folded proteins which carry chemical links, introduced during peptide synthesis, designed to retain the secondary structure in the native protein molecule. Stapled peptides have been investigated as potential modulators of protein–protein interactions for over two decades. The potential use of stapled peptides as inhibitors of viral entry, and therefore as antiviral therapeutics, has been established for several important viruses causing disease in humans, such as the human immunodeficiency virus type 1 (HIV-1), respiratory syncytial virus (RSV), and Middle East Respiratory Syndrome (MERS) coronavirus. Several independent research initiatives have investigated the inhibitory effect of stapled peptides on binding of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, to its receptor, angiotensin-converting-enzyme 2 (ACE2). These stapled peptides, which mimic Helix 1 of the human ACE2 receptor, have demonstrated mixed ability to prevent infection with SARS-CoV-2 in cell-based studies.

KEYWORDS
COVID-19, hACE2, inhibitor, peptide, SARS-CoV-2, stapling, therapeutic

1 | INTRODUCTION

Short peptides, when isolated from the parent protein, lose their secondary structure and exist in solution as unstructured, linear molecules. Loss of secondary structure usually means loss of biological function. Because peptides can lose their shape when taken out of context, developing chemical interventions to stabilize their bioactive structure remains an active area of research.[1] Stapling involves such chemical modifications designed to constrain the peptide chain in the secondary structure of the native conformation, and the peptide products are known as stapled peptides.

Stapling can be applied to enhance the alpha-helicity of peptides, which would otherwise adopt a random coil conformation in solution. Increased helicity renders them more resistant to proteolytic breakdown by host proteases; furthermore, stapling increases potency and often improves cell penetration.[2] Stapling of alpha helices usually involves linking one amino acid residue with the equivalently located residue one turn (residue *i* linked to residue *i*+4) or two turns (residue *i* linked to residue *i*+7) further along the helix. Commonly used linkers are hydrocarbon chains introduced as side chains of unnatural amino acid residues, which are then chemically bonded together during the final stages of peptide synthesis, or side chains of Asp and Glu residues joined to side chains of Arg and Lys residues via lactamization of the side chains.

The general concept of peptide stapling was introduced about two decades ago by Verdine et al.[3] Schafmeister et al. first demonstrated that an all-hydrocarbon cross-linking system could greatly increase the helical propensity and metabolic stability of peptides.[4] This finding was applied to create peptides which interfered with regulation of apoptosis in tumor cells by BCL-2 family proteins and their interaction.[3] Control of apoptosis in BCL-2 proteins is mediated through an alpha-helical segment, BH3. The group of Verdine first used the term "stapled peptides" to describe peptides mimicking the BH3 helical region, which had increased affinity to multidomain BCL-2 member pockets and improved pharmacological properties.[3] One of these stapled peptides specifically activated the apoptotic pathway to kill leukemia cells. These researchers suggested that hydrocarbon stapling of native peptides could prove useful for

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modulation of protein–protein interactions (PPIs) in many signaling pathways.

Stabilization of alpha-helices may be achieved by lactam bridges, disulfide bridges, H-bond surrogates, and alpha/beta peptides.[5–7] However, these alternatives to hydrocarbon stapling involve cross-links that are both polar and pharmacologically labile.[8]

2 | STAPLED PEPTIDES AS MODULATORS OF PPI

Transcription factors, which play a major role in regulation of cell state and neoplastic growth, are considered prime candidates as drug targets.[9] Use of stapled peptides to target the interaction of transcription factors was reported in earlier research of Moellering et al.,[10] who designed hydrocarbon-stapled alpha-helical peptides targeting the critical protein–protein interface in the Notch transcription complex. Treatment of leukemia cells with these hydrocarbon-stapled peptides resulted in genome-wide suppression of Notch-activated genes.[10] Direct antagonism of Notch transcription caused potent, Notch-specific antiproliferative effects in cultured cells and in a mouse model of Notch1-driven T-cell acute lymphoblastic leukemia (T-ALL).[10]

The tumor suppressor p53 protein plays a crucial role in maintaining genome integrity; inactivation of p53 is the most prevalent defect in human cancers. A stapled peptide, ALRN_6924, that activates p53 by preventing its interaction with its negative regulator murine double minute 2 (MDM2) has recently entered clinical trials. This stapled peptide mimics the interaction of p53 with MDM2.[11]

3 | STAPLED PEPTIDES AS INHIBITORS OF HOST CELL ENTRY BY VIRUSES AND THEIR MATURATION

PPIs feature prominently amongst the mechanisms by which viruses enter host cells and by which newly synthesized viral components are assembled prior to release.

The capsid domain of the human immunodeficiency virus type 1 (HIV-1) Gag polyprotein is a critical determinant of virus assembly, and is therefore one such potential drug target for treating AIDS.[12] Viral assembly occurs through controlled polymerization of the Gag polyprotein after its transportation to the site of viral assembly in the host cell membrane. Zhang et al. created a cell-penetrating peptide (CPP) by applying hydrocarbon-stapling to a 12-mer alpha-helical peptide, capsid assembly inhibitor (CAI) that had previously been found to disrupt immature- and mature-like capsid particle assembly in vitro.[12,13] The CPP created by Zhang et al., NYAD-1, was generated by replacing two natural amino acids at the 4 (*i*) and 8 (*i*+4) positions of the CAI sequence by a non-natural amino acid (S)-Fmoc-2-(2′-pentenyl)alanine. NYAD-1 showed enhanced alpha-helicity and cell-penetrating ability and inhibited HIV-1 isolates in cell culture at low micromolar potency, thus indicating its potential for further development as an antiviral drug to treat AIDS.[12]

Prior to viral entry into the host cell, the fusion peptide, F, of the respiratory syncytial virus (RSV) is activated into a metastable pre-fusion state upon cleavage by host cell proteases. The F pre-fusion state contains a hydrophobic fusion peptide, two heptad repeats (HR1 and HR2), and a viral transmembrane domain. Following the insertion of the fusion peptide into the target cell membrane, F undergoes a conformational rearrangement to yield a six-helix bundle hairpin structure, whereby three HR2 sequences bind in an antiparallel manner to a trimeric HR1 coiled coil. The hairpin structure brings the viral and target cell membranes into close proximity, thereby facilitating membrane fusion and subsequent viral entry. Gaillard et al. synthesized short double-stapled peptides which interfered with formation of the six-helix bundle post-fusion structure required for viral cell entry[14] and had potent antiviral activity as well as being resistant to proteolysis. Importantly, intranasal delivery of the most potent stapled peptide significantly decreased RSV infection in the respiratory tract of BALB/c mice.[14]

Coronaviruses also use a six-helix bundle to mediate membrane fusion with the host target cell. However, the length of the N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR) sequences in this six-helix bundle structure differs markedly between different viral families, despite overall topological similarity. Wang et al. employed hydrocarbon stapling to reproduce the topography of alpha-helical motifs found in the Middle East Respiratory Syndrome (MERS) coronavirus CHR regions; they designed double-stapled peptides mimicking helical motifs in the CHRs, which inhibited spike (S) protein-mediated cell–cell fusion and ultimately infection by MERS.[15]

Another example of design of stapled peptides to block viral entry concerns the design of HIV-1 inhibitor stapled peptides. The 36-mer antiretroviral peptide enfuvirtide blocks HIV-1 cell fusion and therefore viral entry into host cells. This 36-mer peptide comprises residues 638–673 of the gp41 envelope glycoprotein, mimicking the heptad repeat 2 (HR2) oligomerization domain, and thereby disrupting assembly of the six-helix bundle viral fusion apparatus.[16]

Enfuvirtide was developed over two decades ago showing significant efficacy as well as good pharmacological and safety data when tested in HIV-1 patients.[17,18] However, enfuvirtide requires high dosing and repeated injections due to its lack of oral bioavailability.[17] The poor in vivo stability and lack of oral bioavailability of enfuvirtide led to its being relegated to a treatment of last resort.[19] Bird et al. optimized HIV-1 fusion inhibitors by applying all-hydrocarbon covalent cross-linking to create so-called "next-generation gp41 HR2 peptides," with markedly increased protease resistance and antiviral activity compared with enfuvirtide.[19]

More recently, Gomara et al.[20] described the design and synthesis of stapled peptides based on the peptide E1P47, which corresponds to residues 184–201 of the envelope glycoprotein E1 of the human pegivirus, GB virus C (UniProt Q69431_9FLAV). Gomara et al. synthesized stapled peptides by lactamization between the amine-side

and carboxy-side chains of Lys and Asp or Glu amino acid residues. Compared with the parent peptide, E1P47, these lactam-stapled peptides showed greater inhibitory potencies in cellular models and inhibited HIV-1 infection in colorectal tissue explants, but they did not show improved resistance to serum proteases.[20]

4 | INHIBITION OF SARS-COV-2 ENTRY INTO HOST CELLS

Several current research initiatives are aimed at investigating the use of stapled peptides as potential inhibitors of host cell entry of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19). Such stapled peptides represent potential preventative and treatment therapies for COVID-19. A brief outline of the mechanism of entry of SARS-CoV-2 into host cells follows. For a detailed understanding of the structural and cell biological foundations of the multistep SARS-CoV-2 entry process, the reader is referred to recent reviews.[21–23]

5 | INTERACTION OF SARS-COV-2 WITH ACE2 RECEPTOR REQUIRED FOR VIRAL ENTRY

The SARS-CoV-2, the causative agent of COVID-19, enters host cells by attaching to the membrane-located angiotensin-converting-enzyme 2 (ACE2).[24] A viral surface spike glycoprotein (S) mediates the entry of coronavirus into host cells. S protein is assembled as a homotrimer and is inserted in multiple copies into the membrane of the virion (virus particle). Each SARS-CoV-2 virion has about 24–40 S proteins distributed randomly over its entire surface, resulting in the distinctive crown-like appearance seen in electron micrographs[23,25] (Figure 1). Many other viruses have proteins on their surfaces, similar to the S protein, which mediate cell entry and host infection, such as influenza hemagglutinin and the envelope glycoproteins of HIV-1 and Ebola virus. It is the S glycoprotein of SARS-CoV-2 that starts the process of infection, binding to host cell receptors and subsequently fusing with the host cell membrane to release the viral genome inside the cell. A discrete region of the S glycoprotein, the so-called receptor-binding domain (RBD), is responsible for interaction with the ACE2 receptor. The RBD of the S glycoprotein binds with the peptidase domain (PD) of the ACE2 receptor and triggers membrane fusion followed by entry of the virus into host cells. The RBD has to be in the so-called "up" position, exposing the receptor-binding motif (RBM), before it can engage with the ACE2 receptor (Figure 2). The S glycoprotein of SARS-CoV-2 is cleaved by proprotein convertases such as furin into S1 and S2 subunits during their biosynthesis in the Golgi apparatus in infected, virus-producer cells. Thus, in the mature virion, S1 and S2 exist as two non-covalently associated subunits. Host cell entry requires S protein cleavage at the S1/S2 junction as well as at a site in the S2 unit, S2′.[27] The host cell protease, transmembrane protease serine 2 (TMPRSS2) cleaves the S glycoprotein of SARS-CoV-2 at the junction of S1 and S2 as well as at the S2′ site.[27]

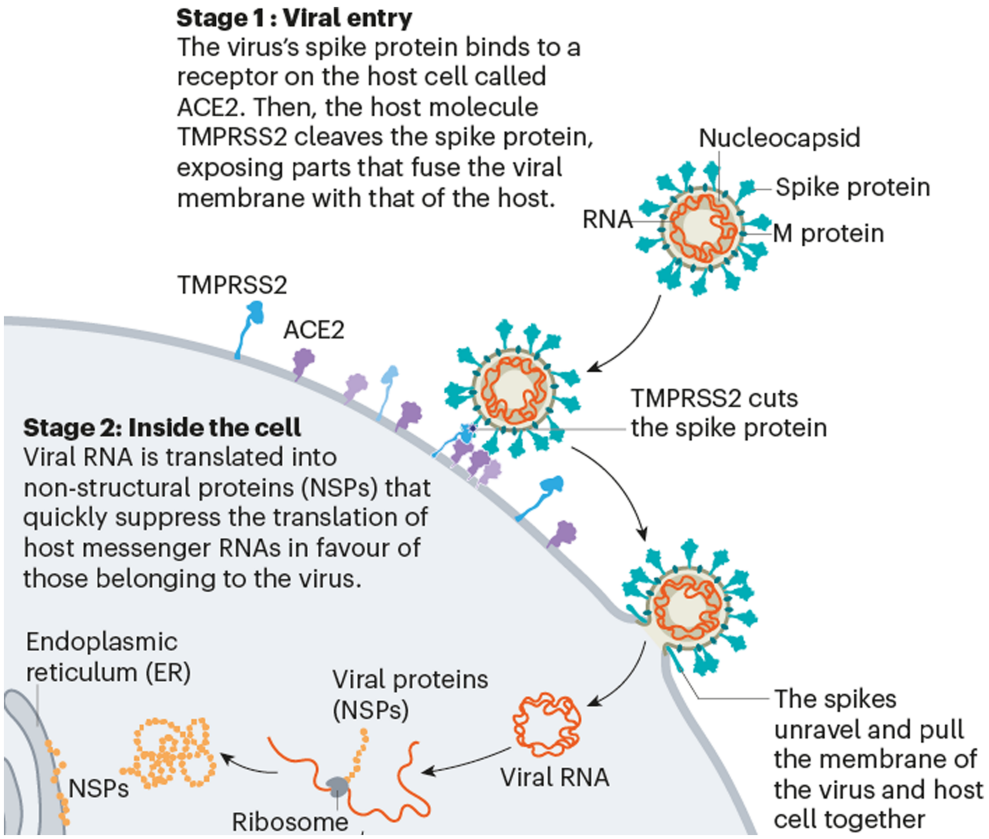

FIGURE 1 Simplified schematic to illustrate how severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) enters host cells (adapted with permission from Scudellari M, "How the coronavirus infects cells—And why Delta is so dangerous", Nature, 2021, Springer Nature B.V. [23])

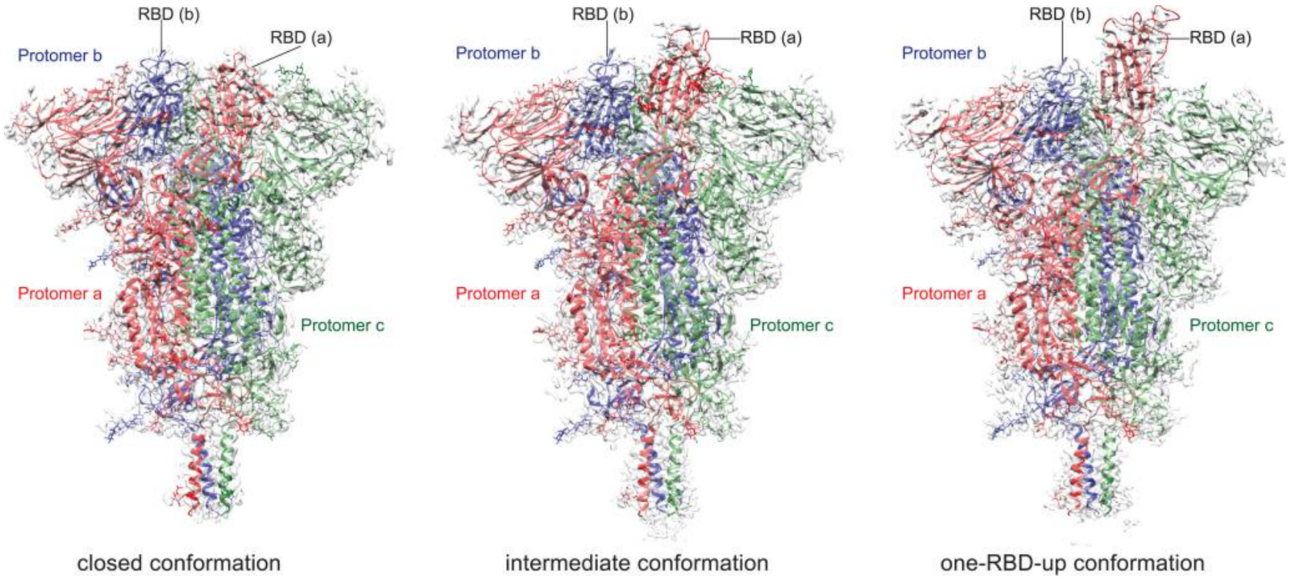

FIGURE 2 Cryo-EM structures of the full-length severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) S protein carrying G614. Three structures of the G614 S trimer representing a closed, three "receptor-binding domain (RBD) down" conformation, an RBD intermediate conformation and a one "RBDup" conformation modeled on the basis of corresponding cryo-EM density maps at 3.1- and 3.5-Å resolution (adapted with permission from Zhang J, "Structural impact on SARS-CoV-2 spike protein by D614G substitution", Science, 2021, John Wiley & Sons[26])

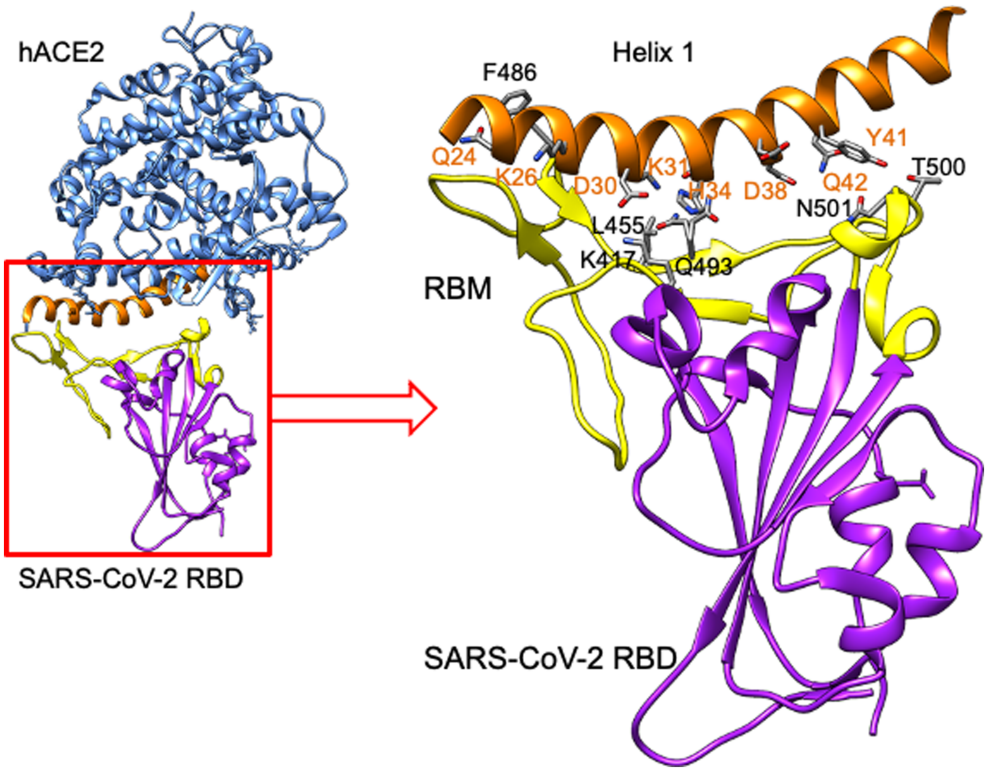

FIGURE 3 X-ray structure (PDB ID:6m0j) showing binding of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) receptor-binding domain (RBD) (purple), including the interacting receptor binding motif (RBM) (yellow) with the hACE2 receptor (blue). Detailed interactions of helix 1 of hACE2 (orange) with the SARS-CoV-2 RBD are magnified in the illustration on the right.[28] Residues in hACE2 colored orange, in the SARS-CoV-2 RBD, black. Molecular graphics performed with UCSF Chimera, version 1.14[29]

The S1 subunit contains the RBD and therefore initiates direct binding with the ACE2 PD; the S2 subunit anchors the S glycoprotein to the membrane and carries a fusion peptide, heptad repeats HR1 and HR2, and other machinery necessary for membrane fusion.[22]

The critical binding region on the ACE2 receptor molecule is a helical region about 30 amino acid residues in length called Helix 1 (or alpha1 helix) (Figure 3). ACE2 engagement by the virus exposes the S2′ site; S2′ site cleavage by TMPRSS2 at the cell surface or

cathepsin L in the endosomal compartment following ACE2-mediated endocytosis ensues and is followed by release of the fusion peptide. Thus, TMPRSS2-mediated S protein activation occurs at the plasma membrane, whereas cathepsin-mediated activation occurs in the endolysosome. Cleavage of S2′ provides the trigger for massive conformational change in the S1 and S2 subunits, which results in viral and host membranes coming into close proximity. The C-terminal region of HR2 forms a long helix and folds into a six-helix bundle structure with the rest of the HR1 coiled coil; in this way, a stable, rigid post-fusion structure is formed. A fusion pore is created that allows the viral genome to reach the host cell cytoplasm.[22]

Recently, soluble human ACE2 (hACE2) was proposed as a competitive interceptor (decoy receptor) of SARS-CoV-2 and therefore as a potential therapy for COVID-19.[30] A recombinant soluble hACE2 (rhACE2) has recently completed Phase II clinical trials.[31] However, in both humans and mice, recombinant ACE2 has a high clearance rate, with a plasma half-life of a few hours in pharmacokinetic studies.[32,33] This, coupled with the high cost of manufacture, means that a more desirable solution would be to design smaller molecules and focus exclusively on the precise region of the hACE2 molecule which interacts with the viral S protein.[34]

6 | STAPLED PEPTIDES AS INHIBITORS OF HOST CELL ENTRY BY SARS-COV-2: ONGOING RESEARCH

Stapled peptides being investigated by several research groups as potential inhibitors of the SARS-CoV-2 interaction with its hACE2 target are modeled on the critical binding region of hACE2, the helical region, Helix 1, which binds SARS-CoV-2 (Figures 3 and 4). The underlying principle of these peptidomimetics is that they act as decoys of the hACE2 binding site for SARS-CoV-2, diverting the virus from initial binding to the receptor, thereby preventing virus entry and subsequent infection.[2]

Curreli et al.[2] synthesized double-stapled peptides by solid-phase peptide synthesis (SPPS), using non-natural amino acids to introduce alkene hydrocarbon staples to Helix 1 of hACE2 (Figure 4). Peptides were designed to incorporate either *i*, *i*+7 or *i*, *i*+4 hydrocarbon-staples that would conformationally constrain the peptide over approximately two turns or one turn of the helix, respectively. These researchers evaluated the anti-SARS-CoV-2 activity of four double-stapled peptides in cell-based assays. To do this, they pretreated SARS-CoV-2 pseudovirus with increasing concentrations

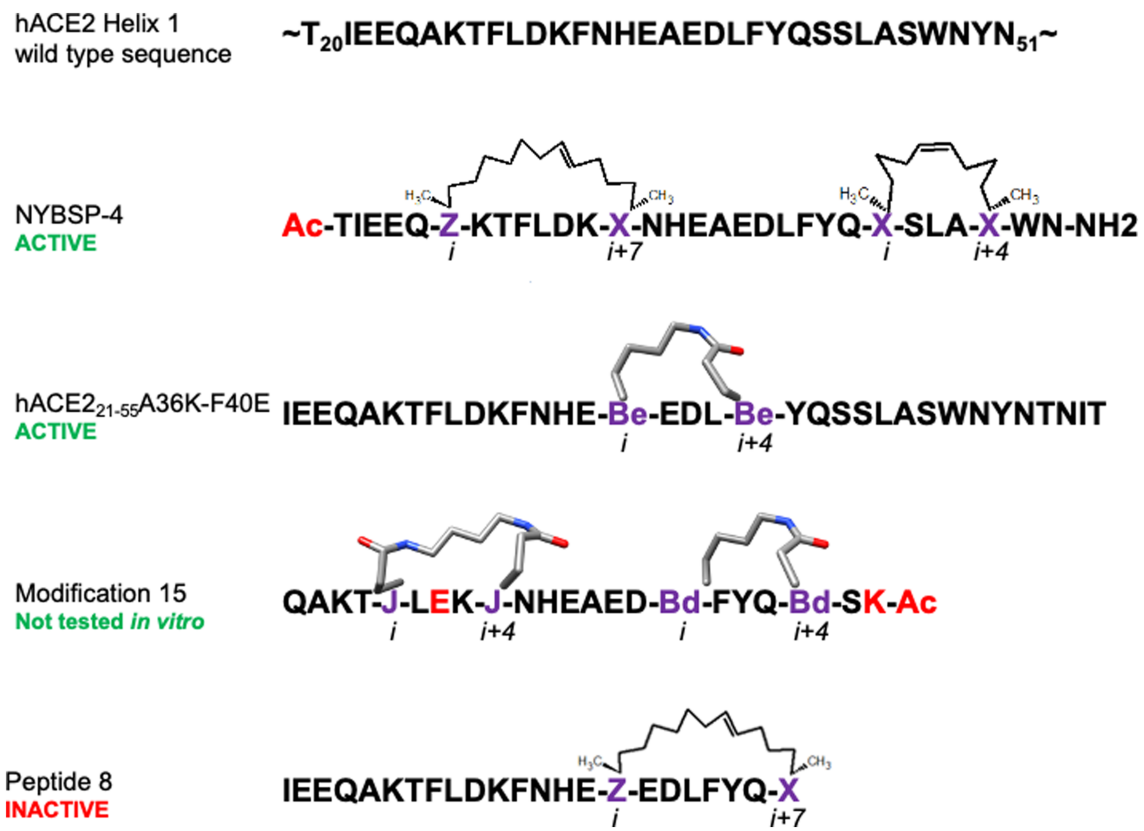

FIGURE 4 Stapled peptides based on Helix 1 of hACE2 (UniProt: Q9BYF1, ACE2_HUMAN). Peptides NYBSP-4[2] and hACE2$_{21\text{-}55}$A36K-F40E[35] inhibited entry of SARS-CoV-2 in cell-based assays. Peptide Modification 15[36] showed binding to SARS-CoV-2 RBD in silico with similar docking scores to NYBSP-4. Peptide 8[37] did not inhibit entry of SARS-CoV-2 in cell-based assays. Be, lactam bridge formed by Lys-Glu side chain bonding; Bd, lactam bridge formed by Lys-Asp side chain bonding; J, double lactam bridge formed by Glu-Orn-Glu bonding; X, *S*-2-(4-pentenyl)alanine; Z, *R*-2-(7-octenyl)alanine. Residues and chemical groups shown in red differ from wild-type sequence. Images of staples produced using UCSF Chimera, version 1.14[29] and ChemSketch

of the peptides and then infected two different cell lines overexpressing hACE2: human fibrosarcoma HT1080/ACE2 cells and human lung carcinoma A549/ACE2 cells, with the pretreated virus. As negative controls, they used the equivalent Helix 1 linear peptide and an unrelated double-stapled peptide. Three of the four hACE2 stapled peptides showed an inhibitory effect on pseudovirus infection of the cells, with IC_{50} values in the low micromolar range, whereas the controls, as expected, had no antiviral activity. The same three peptides exhibited moderate antiviral activity against SARS-CoV-2 in a microneutralization assay in Vero cells. Furthermore, the peptides all had a plasma half-life of greater than 289 min, indicating fairly good resistance to proteolytic breakdown by host enzymes in human plasma. Circular dichroism (CD) spectroscopy was used to determine the average helicity of the peptides which was found to range from 50% to 94%. The best performing peptide, NYBSP-4, with helicity 80% showed the highest antiviral activity against SARS-CoV-2 pseudovirus in human fibrosarcoma HT1080 cells overexpressing ACE2, with an IC_{50} of 1.97 μM.[2] Further modification to improve their plasma half-lives and potency would make these peptides attractive therapeutic candidates for preclinical studies.

De Campos et al.[36] employed rational structure-based design to propose 22-mer stapled peptides using the structure of the hACE2 Helix 1, as a template. In this in silico approach, the researchers used different types of molecular configurations to construct the chemical linkers: lactam bridge, double lactam bridge, and alkene link (Figure 4). The peptides were designed to retain the alpha-helical character of the natural structure of Helix 1, to enhance binding affinity, and to have better solubility compared with analogous peptides designed by other research groups.[36] The hydrophobicity of the peptides was reduced compared with the native Helix 1 by replacing hydrophobic residues at the position of the staples with residues of a more hydrophilic nature. Other amino acid residues in the natural sequence were replaced to optimize interactions with the RBD of SARS-CoV-2. Interaction of the peptides with SARS-CoV-2 was investigated in docking experiments using the crystal structure of hACE2 bound to the RBD of SARS-CoV-2 (PDB ID: 6M0J). The most promising peptides from these docking experiments were then subjected to molecular dynamics simulations in order to analyze the stability and dynamic properties of interaction. Two distinct peptides, Modification 11 and Modification 15 (Figure 3), emerged as the best candidates from two different docking protocols. Importantly, the top-ranked stapled peptides presented docking scores similar to the docking score obtained with NYBSP-4, the most promising peptide created by the group of Curreli et al., and experimentally proven to inhibit binding of the RBD of SARS-CoV-2 to hACE2 in cell-based assays. Modification 11 and Modification 15 have yet to be synthesized and tested in vitro.

Maas et al.[35] designed and synthesized lactam-based, single-stapled 35-mer peptides based on the hACE2 N-terminal Helix 1, in which they avoided placing the staples at certain polar residue positions known from computational studies to interact directly with the SARS-CoV-2 RBD. These researchers synthesized (by SPPS) peptides with a single *i*, *i*+4 staple, at one of the residue positions, F28-F32 ($hACE2_{21\text{-}55}$F28K-F32E), F32-A36 ($hACE2_{21\text{-}55}$F32K-A36E), or A36-F40 ($hACE2_{21\text{-}55}$A36K-F40E) (Figure 4). As controls, they synthesized the equivalent linear modified peptides with lysine and glutamic acid residues replacing the wild-type residues, but lacking the lactam link.[35] Alpha-helical content of the synthetic hACE2 peptides was confirmed by CD spectroscopy. The average helicity of the peptides ranged from 11% to 52%. Stapled peptide $hACE2_{21\text{-}55}$A36K-F40E had the highest average helicity of 52%, compared with the wild-type sequence 38%. Peptides were tested for their ability to inhibit binding of the virus RBD in an immunoassay, using a concentration–response curve of SARS-CoV-2 S protein (RBD) recombinant human monoclonal antibody as a positive control. Peptide $hACE2_{21\text{-}55}$A36K-F40E showed significant inhibition of binding (IC_{50}: 3.6 μM) compared with the linear wild-type control that displayed only minor inhibition at concentrations greater than 100 μM. Resistance of the peptides to proteolysis by serum proteases was determined, recording peptide degradation in serum: PBS (1:4 v/v) over time using analytical high-performance liquid chromatography (HPLC). Peptide $hACE2_{21\text{-}55}$A36K-F40E and, interestingly, the wild-type peptide were very stable, showing less than 20% degradation after 3 h. Thus, stapled peptide $hACE2_{21\text{-}55}$A36K-F40E, which has increased helicity, efficiently inhibits SARS-CoV-2 S protein-hACE2 binding and is resistant to degradation in the serum, seems to offer potential for continued optimization as a COVID-19 therapeutic (Figure 4).

In contrast to the above three studies, which produced promising results, a fourth research group failed initially to show satisfactory binding of their stapled peptides modeled on Helix 1 of hACE2.[37] These researchers initially synthesized a range of 22-mer peptidomimetic analogues with single *i*, *i*+7 or *i*, *i*+4 staples for comparison with the native Helix 1 peptide. A histidine residue (H34) is located at a bend the middle of Helix 1. Staples were placed on either side of H34, as well as across it, to test the importance of this bend within the helix. Truncated analogues were also designed, based on the residues that interact with the RBD. Notably, these peptides incorporated the same unnatural amino acids as used in the alkene-stapled peptides of the Curreli group, namely, *S*-2-(4-pentenyl)alanine and *R*-2-(7-octenyl)alanine (Figure 4). Peptide 8 (Figure 4) showed 72% helicity. However, none of these peptides inhibited viral entry in cell-based assays.

Following these negative results, Morgan et al.[37] designed second-generation, longer peptidomimetics of hACE2 Helix 1. One of these peptides, in contrast to the first-generation peptides, did show potent activity (IC_{50} 100 nM) in a SARS-CoV-2 pseudovirus neutralization assay, but the other peptides were inactive.

7 | CONCLUSION AND DISCUSSION

The research initiatives described herein target initial recognition of the hACE2 receptor in the membrane of host cells, by the RBD in the S glycoprotein of SARS-CoV-2, for identifying potential therapeutics to inhibit viral entry. All use stapled peptides as peptidomimetics to act as decoys of Helix 1 of hACE2, where initial binding of S protein to receptor occurs (Figure 5). The underlying principle of applying

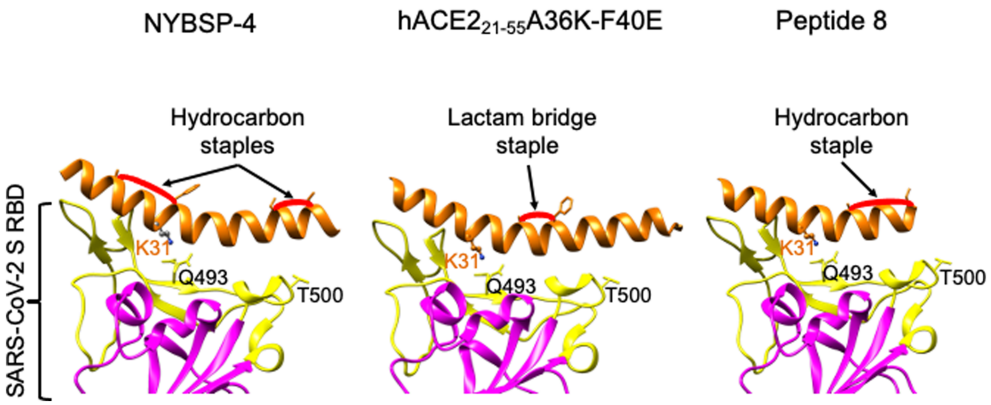

FIGURE 5 Graphic to show different stapling positions in peptides NYBSP-4,[2] hACE221-55A36K-F40E,[35] and Peptide 8,[37] modeled in the context of the crystal structure of the SARS-CoV-2 spike receptor-binding domain (RBD) bound to the ACE2 receptor (PDB ID:6m0j).[28] Residues important for binding labeled in peptides, K31 (orange), and in SARS-CoV-2 spike RBD, Q493 and T500 (black). Coloring as in Figure 3. Molecular graphics performed with UCSF Chimera, version 1.14[29]

TABLE 1 Comparison of results for best-performing stapled peptides tested in vitro for antiviral activity against SARS-CoV-2 pseudovirus

Stapled peptide	IC_{50} (μM)	Plasma half-life (min)	Helicity (%)	Reference
NYBSP-4	1.97[a]	>289	80	Curreli, 2020[2]
hACE2$_{21-55}$A36K-F40E	3.60[b]	>180	52	Maas, 2021[35]

[a]Peptide tested in human fibrosarcoma HT1080 cells overexpressing ACE2.
[b]Peptide tested in an immunoassay.

stapling in all these efforts is to retain the bioactive secondary structure of Helix 1. Although the stapling resulted in successful retention of helicity, a directly proportional relationship of helicity to antiviral activity was not observed. For instance, the average helicity of the stapled peptides of Curreli et al. ranged from 50% to 94%; their best performing peptide, NYBSP-4, however had a helicity of 80% and showed the highest antiviral activity against SARS-CoV-2 pseudovirus in human fibrosarcoma HT1080 overexpressing ACE2, with an IC_{50} of 1.97 μM.[2] Peptide 8 (Figure 3) of Morgan et al. was inactive, yet showed 72% helicity (Figure 4, Table 1).[37]

The SARS-CoV-2 spike protein is a homotrimer, carrying three RBDs each of which can bind to an hACE2 receptor molecule. Peptidomimetics mimicking Helix 1 of hACE2 would have to be present in very high concentration to outcompete binding of the SARS-CoV-2 virus to the hACE2 receptor in the host cell membrane. Moreover, initial binding of a peptidomimetic of Helix 1 to the RBD on one protomer of a spike homotrimer molecule, might cause release and exposure of the RBD on a second protomer of the homotrimer, resulting in an activation rather than an inhibition of viral entry. Such considerations might explain the mixed results obtained in the experiments with stapled peptides mimicking Helix 1 of hACE2 described here.

The strategy of prevention of viral entry by these Helix 1 peptidomimetics, differs from that of previous work with stapled peptides used to prevent viral entry of other viruses, in that the latter have mostly aimed at interfering with the fusion mechanism, subsequent to initial recognition, whereby the viral and host cell membranes fuse together, permitting viral entry into host cells. An example previously mentioned, the 36-mer peptide, anti-HIV-1 drug, enfuvirtide, mimics the heptad repeat 2 (HR2) oligomerization domain and disrupts assembly of the six-helix bundle viral fusion apparatus.[16]

Stapled peptides, if used as inhibitors of SARS-CoV-2 entry, would have to be applied very early on in any infection—ideally before symptoms of infection appear. An important consideration for developing useful inhibitors of viral entry is the initial entry point of the virus. For SARS-CoV-2, the major route of infection, where most replication initially takes place, is in the lower and upper airways. Stapled peptides applied as antiviral drugs would need to be delivered to and exist stably on the respiratory epithelia. Interaction of SARS-CoV-2 S RBD with membrane-bound hACE2 in the pulmonary epithelium could be targeted by inhalation of an aerosolized peptide solution. An inhalatory or intranasal route for delivery would deliver the peptide drug directly to its target in the alveolar and bronchial, nasal, and pharyngeal epithelia, thereby circumventing the problem of reduced plasma half-life.

Taken together, results of research efforts to investigate the potential of stapled peptides mimicking the hACE2 receptor recognition site as potential inhibitors of SARS-CoV-2 entry into host cells showed mixed results. More knowledge of the precise sequence of events surrounding recognition of the receptor by the RBD binding site in the spike protein of SARS-CoV-2 is required to determine which features of the stapled peptides are important for inhibition of the recognition event. Nevertheless, some stapled peptides have shown an inhibitory effect on host cell entry by SARS-CoV-2 pseudovirus in cell-based experiments, with IC_{50} values in the low micromolar

and high nanomolar range. This would seem to indicate that the quest for stapled peptides, with improved potency, as COVID-19 therapeutics is worth pursuing.[38]

ORCID

Susan Tzotzos https://orcid.org/0000-0003-4802-707X

REFERENCES

1. Walensky LD, Bird GH. Hydrocarbon-stapled peptides: principles, practice, and progress. *J Med Chem*. 2014;57(15):6275-6288. doi:10.1021/jm4011675
2. Curreli F, Victor SMB, Ahmed S, et al. Stapled peptides based on human angiotensin-converting enzyme 2 (ACE2) potently inhibit SARS-CoV-2 infection in vitro. *MBio*. 2020;11(6):e02451-20. doi:10.1128/mBio.02451-20
3. Walensky LD, Kung AL, Escher I, et al. Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. *Science*. 2004;305(5689):1466-1470. doi:10.1126/science.1099191
4. Schafmeister CE PJ, Verdine GL. An all-hydrocarbon cross-linking system for enhancing the helicity and metabolic stability of peptides. *J am Chem Soc*. 2000;122(24):5891-5892. doi:10.1021/ja000563a
5. Wang D, Chen K, Kulp Iii JL, Arora PS. Evaluation of biologically relevant short α-helices stabilized by a main-chain hydrogen-bond surrogate. *J Am Chem Soc*. 2006;128(28):9248-9256. doi:10.1021/ja062710w
6. Boersma MD, Haase HS, Peterson-Kaufman KJ, et al. Evaluation of diverse α/β-backbone patterns for functional α-helix mimicry: analogues of the Bim BH3 domain. *J Am Chem Soc*. 2012;134(1):315-323. doi:10.1021/ja207148m
7. Ali AM, Atmaj J, Van Oosterwijk N, Groves MR, Domling A. Stapled peptides inhibitors: a new window for target drug discovery. *Comput Struct Biotechnol J*. 2019;17:263-281. doi:10.1016/j.csbj.2019.01.012
8. Blackwell HE, Grubbs RH. Highly efficient synthesis of covalently cross-linked peptide helices by ring-closing metathesis. *Angew Chem Int Ed Engl*. 1998;37(23):3281-3284. doi:10.1002/(SICI)1521-3773(19981217)37:23%3C3281::AID-ANIE3281%3E3.0.CO;2-V
9. Darnell JE Jr. Transcription factors as targets for cancer therapy. *Nat Rev Cancer*. 2002;2(10):740-749. doi:10.1038/nrc906
10. Moellering RE, Cornejo M, Davis TN, et al. Direct inhibition of the NOTCH transcription factor complex. *Nature*. 2009;462(7270):182-188. doi:10.1038/nature08543
11. Kannan S, Partridge AW, Lane DP, Verma CS. The dual interactions of p53 with MDM2 and p300: implications for the design of MDM2 inhibitors. *Int J Mol Sci*. 2019;20(23):20 doi:10.3390/ijms20235996
12. Zhang H, Zhao Q, Bhattacharya S, et al. A cell-penetrating helical peptide as a potential HIV-1 inhibitor. *J Mol Biol*. 2008;378(3):565-580. doi:10.1016/j.jmb.2008.02.066
13. Sticht J, Humbert M, Findlow S, et al. A peptide inhibitor of HIV-1 assembly in vitro. *Nat Struct Mol Biol*. 2005;12(8):671-677. doi:10.1038/nsmb964
14. Gaillard V, Galloux M, Garcin D, et al. A short double-stapled peptide inhibits respiratory syncytial virus entry and spreading. *Antimicrob Agents Chemother*. 2017;61(4):e02241-16. doi:10.1128/AAC.02241-16
15. Wang C, Xia S, Zhang P, et al. Discovery of hydrocarbon-stapled short α-helical peptides as promising Middle East Respiratory Syndrome Coronavirus (MERS-CoV) fusion inhibitors. *J Med Chem*. 2018;61(5):2018-2026. doi:10.1021/acs.jmedchem.7b01732
16. Kilby JM, Hopkins S, Venetta TM, et al. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nat Med*. 1998;4(11):1302-1307. doi:10.1038/3293
17. Kilby JM, Lalezari JP, Eron JJ, et al. The safety, plasma pharmacokinetics, and antiviral activity of subcutaneous enfuvirtide (T-20), a peptide inhibitor of gp41-mediated virus fusion, in HIV-infected adults. *AIDS Res Hum Retroviruses*. 2002;18(10):685-693. doi:10.1089/088922202760072294
18. Lazzarin A, Clotet B, Cooper D, et al. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. *N Engl J Med*. 2003;348(22):2186-2195. doi:10.1056/NEJMoa035211
19. Bird GH, Madani N, Perry AF, et al. Hydrocarbon double-stapling remedies the proteolytic instability of a lengthy peptide therapeutic. *Proc Natl Acad Sci U S A*. 2010;107(32):14093-14098. doi:10.1073/pnas.1002713107
20. Gomara MJ, Perez Y, Gomez-Gutierrez P, et al. Importance of structure-based studies for the design of a novel HIV-1 inhibitor peptide. *Sci Rep*. 2020;10(1):14430 doi:10.1038/s41598-020-71404-0
21. Wang Y, Grunewald M, Perlman S. Coronaviruses: an updated overview of their replication and pathogenesis. *Methods Mol Biol*. 2020;2203:1-29. doi:10.1007/978-1-0716-0900-2_1
22. Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into cells. *Nat Rev Mol Cell Biol*. 2022;23(1):3-20. doi:10.1038/s41580-021-00418-x
23. Scudellari M. How the coronavirus infects cells—and why Delta is so dangerous. *Nature*. 2021;595(7869):640-644. doi:10.1038/d41586-021-02039-y
24. Shang J, Ye G, Shi K, et al. Structural basis of receptor recognition by SARS-CoV-2. *Nature*. 2020;581:221-224.
25. Ke Z, Oton J, Qu K, et al. Structures and distributions of SARS-CoV-2 spike proteins on intact virions. *Nature*. 2020;588(7838):498-502. doi:10.1038/s41586-020-2665-2
26. Zhang J, Cai Y, Xiao T, et al. Structural impact on SARS-CoV-2 spike protein by D614G substitution. *Science*. 2021;372(6541):525-530. doi:10.1126/science.abf2303
27. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*. 2020;181(2):271-280. doi:10.1016/j.cell.2020.02.052
28. Lan J, Ge J, Yu J, et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature*. 2020;581(7807):215-220. doi:10.1038/s41586-020-2180-5
29. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera? A visualization system for exploratory research and analysis. *J Comput Chem*. 2004;25(13):1605-1612. doi:10.1002/jcc.20084
30. Batlle D, Wysocki J, Satchell K. Soluble angiotensin-converting enzyme 2: a potential approach for coronavirus infection therapy? *Clin Sci (Lond)*. 2020;134(5):543-545. doi:10.1042/CS20200163
31. Bhullar KS, Drews SJ, Wu J. Translating bioactive peptides for COVID-19 therapy. *Eur J Pharmacol*. 2021;890:173661 doi:10.1016/j.ejphar.2020.173661
32. Wysocki J, Ye M, Rodriguez E, et al. Targeting the degradation of angiotensin II with recombinant angiotensin-converting enzyme 2. *Hypertension*. 2010;55(1):90-98. doi:10.1161/HYPERTENSIONAHA.109.138420
33. Haschke M, Schuster M, Poglitsch M, et al. Pharmacokinetics and pharmacodynamics of recombinant human angiotensin-converting enzyme 2 in healthy human subjects. *Clin Pharmacokinet*. 2013;52(9):783-792. doi:10.1007/s40262-013-0072-7
34. Cao L, Goreshnik I, Coventry B, et al. De novo design of picomolar SARS-CoV-2 miniprotein inhibitors. *Science*. 2020;370(6515):426-431. doi:10.1126/science.abd9909
35. Maas MN, Hintzen JCJ, Loffler PMG, Mecinovic J. Targeting SARS-CoV-2 spike protein by stapled hACE2 peptides. *Chem Commun (Camb)*. 2021;57(26):3283-3286. doi:10.1039/D0CC08387A
36. de Campos LJ, Palermo NY, Conda-Sheridan M. Targeting SARS-CoV-2 receptor binding domain with stapled peptides: an in silico study. *J Phys Chem B*. 2021;125(24):6572-6586. doi:10.1021/acs.jpcb.1c02398

37. Morgan DC, Morris C, Mahindra A, et al. Stapled ACE2 peptidomimetics designed to target the SARS-CoV-2 spike protein do not prevent virus internalization. *Pept Sci (Hoboken)*. 2021;113(4): e24217. doi:10.1002/pep2.24217
38. Schoeman D, Fielding BC. Human coronaviruses: counteracting the damage by storm. *Viruses*. 2021;13(8):1457. doi:10.3390/v13081457

AUTHOR BIOGRAPHY

Susan Tzotzos is a molecular biologist and science writer with professional experience in peptide drug design and development.

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