Development of Antiviral Peptidomimetics

Songyi Xue
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Development of Antiviral Peptidomimetics

by

Songyi Xue

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Date of Approval:
August 24, 2023

Keywords: Helical mimetics, γ-AApeptide, Combinatory library, Protein-protein interaction, Fusion inhibitor, Foldamers

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Dedication

To my beloved mom, dad, girlfriend, and my families' support.
Acknowledgments

Above all, I want to convey my sincere gratitude to Prof. Jianfeng Cai. He kindly accepted me into his group even though my primary areas of interest throughout my undergraduate studies were chemical engineering and polymer chemistry. He has also consistently been very patient with me when I have had challenges during research or when I have made extremely stupid mistakes. In addition to teaching me about theory and experiments, he also taught me how to carry out independent research in the future. During my five years of training in Prof. Cai's group, I firmly convinced myself that my dream is to be an independent faculty and that I can achieve that dream. Without his generous assistance, I would not have been able to complete such wonderful projects or earn my Ph. D degree. Looking back, I can say that deciding to join Prof. Cai's group was the best choice I ever made.

Next, I extend my heartfelt appreciation to the members of my thesis committee, Prof. Kirpal Bisht, Prof. Wayne Guida, and Prof. Feng Cheng. In the meetings for the original research proposal and the data presentation, each of you offered me an abundance of suggestions. These suggestions helped me overcome a lot of difficulties during my research.

Additionally, I am also appreciative of my collaborators from Fudan University, Prof. Shibo Jiang, Prof. Lu Lu, Prof. Wei Xu, and Dr. Xinling Wang. I am unable to complete these beautiful projects without them.

Furthermore, I would like to thank all the past and present Prof. Cai’s group members; it has really been a privilege to work with them.
Finally, I want to acknowledge the unwavering support and understanding of my parents and girlfriend throughout this challenging academic journey. Their love and encouragement have been my constant motivation.
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Abstract

Protein-protein interactions (PPIs) are essential for biological processes and are associated with a number of diseases, including cancer, infectious diseases, and neurodegenerative diseases. As a result, modulation of PPIs has been recognized as one of the most promising strategies to develop the novel drugs. Peptide modulators always exhibit higher specificity and affinities with targets than small compounds or monoclonal antibodies, but their broad medicinal effectiveness is constrained by their poor bioavailability and biostability. Peptidomimetics, which have been developed to mimic the structure as well as function of bioactive peptides and proteins, have shown excellent potential in protein surface mimicry and recognition, modulation of PPIs, catalysis, and other fields. In comparison to conventional α-helixes, these peptidomimetics with unique and unnatural monomers showed enhanced bioavailability and chemodiversity as well as higher resistance to proteolytic degradation. Inspired by this concept, our group had developed a new peptidomimetics-γ-AApeptide, which could fold into well-defined protein-like secondary and tertiary structures, and they display remarkable biological potential for the recognition of protein and nucleic acids. As a result, γ-AApeptides could be developed into useful tools and drug candidates that probe or modulate medicinally relevant cellular processes.

In this study, we describe the development of bioactive antiviral peptidomimetics based on γ-AApeptides. First, we identified a new pan-coronavirus fusion inhibitor to SARS-CoV-2 using the one-bead-two-compound macrocyclic γ-AApeptides combinatorial library. According to this study, combinatorial libraries provide an excellent platform for the development of antiviral inhibitors and other protein-protein interactions’ inhibitors. Second, using the rational design
strategy, we developed a series of helical mimetic sulfonyl-γ-AApeptides to prevent the fusion process of diverse viruses. These two investigations suggested that it is an extremely promising strategy to the development of new drugs by using of L-sulfonyl-AApeptides or D-sulfonyl-AApeptides to modulate a range of protein-protein interactions. A number of applications of γ-AApeptides in the biological field were further supported by all of these investigations.
Chapter 1 Introduction

Note to Reader

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1.1 The chemical structure of γ-AApeptides and sulfonyl-γ-AApeptides

We initially developed γ-AApeptides (γ-substituted-N-acylated-N-aminoethyl amino acids) as a new type of peptidomimetics, which was derived from the γ-chiral PNA scaffold.1,2 They demonstrated remarkable resistance to proteolytic degradation as well as amenability to chemical diversification, enabling them suitable candidates for various biological applications.3,4 Sulfonyl-γ-AApeptides (Figure 1A), a subclass of γ-AApeptides, were found to not only maintain the advantage of γ-AApeptides, but also adopt the well-defined helical conformations (Figure 1B).5

1.2 The crystal structures of sulfonyl-γ-AApeptides

We initially discovered that the heterogeneous 2:1 α/D-sulfonyl-γ-AA hybrid oligomers and heterogeneous 1:1 α/L-sulfonyl-γ-AA hybrid oligomers adopt right-handed 4.5_{16-14} helical conformation and right-handed 4_{13} helices, respectively, based on their crystal structures.7,8 These results showed that peptidomimetics containing sulfonyl-γ-AApeptides units and α-residues could form distinctive heterogeneous foldamers, which prompted us to believe that homogeneous sulfonyl-γ-AApeptides comprising completely unnatural sulfonyl-γ-AApeptides units could also adopt defined folding conformation. Soon later, we successfully obtained the crystal structures of
homogeneous L-sulfonyl-γ-AApeptides and confirmed that this class of foldamers did form the well-defined left-handed 4_14-helix configuration with a radius of 2.8 Å, a helical pitch of 5.1 Å and four side chains per turn (Figure 1C and 1D). The folding propensity of homogeneous L-sulfonyl-γ-AApeptides is stable with left-handed 4_14-helix configuration regardless of side-chain identity. It is noted that helical sulfonyl-γ-AApeptides bear a similar helical pitch to α-peptides (5.4 Å). Moreover, as the helicity of sulfonyl-γ-AApeptides is stabilized by both intramolecular hydrogen bonding and intrinsic curvature of sulfonamide moieties on the molecular backbone, the sulfonyl-γ-AApeptides demonstrated more robust helical stability than α-peptides of the same lengths in solution. Furthermore, the helical handedness of sulfonyl-γ-AApeptide helices could be manipulated by switching the chirality of chiral side chains in the sulfonyl-γ-AApeptide sequences. Therefore, sulfonyl-γ-AApeptide foldamers could be rationally designed to mimic the structure and function of α-helix for biomolecular recognition and modulation of medicinally relevant PPIs.

1.3 The application of homogenous sulfonyl-γ-AApeptides

Based on atomic structures, high stability, and the robust helical folding propensity of sulfonyl-γ-AApeptides, we set out to employ this class of foldamer as the protein helical domain mimetics to modulate a few well-known PPIs. As sulfonyl-γ-AApeptides demonstrated a similar helical pitch to the α-peptides, and have exactly four side chains per turn which is also analogous to those of α-helix (3.6), the side chains of each helical face (e. g. 1a, 3a, 5a, 7a of Figure 1) of sulfonyl-γ-AApeptides could mimic the side chains of i, i+4, i+7 on α-helix. To this end, we designed a series of homogenous sulfonyl-γ-AApeptides which could project similar functional side chains to those crucial residues of p53 helical domain involved in the interaction with MDM2.
It turned out that a series of sequences could tightly bind to MDM2 and disrupt MDM2/p53 effectively, with the most potent sequence PS10 causing the analogous chemical shifts of MDM2 upon binding compared to those induced by p53, suggesting that sulfonyl-γ-AApeptides assumed a similar binding mode to p53. Encouraged by the findings, we moved to another PPI - β-catenin/BCL9 PPI, in which the α-helical HD2 domain of BCL9 is involved in the binding to β-catenin. The Wnt/β-catenin signaling pathway plays an important role in directing cell proliferation, differentiation, and survival, and this signaling pathway depends on the formation of β-catenin supercomplexes with BCL9 or BCL9-like proteins. Thus, disrupting the interaction between β-catenin and BCL9 is a promising strategy to develop anti-cancer agents. However, this PPI is considerably more challenging to inhibit than p53/MDM2 PPI because both hydrophobic and charged residues in BCL9 are important for the recognition of β-catenin. Nonetheless, we successfully mimicked this α-helix region of BCL9 with sulfonyl-γ-AApeptides, and demonstrated sulfonyl-γ-AApeptides were highly cell-permeable and interacted with β-catenin specifically in the cell-based assay. Not long ago, we adopted the design strategy of sulfonyl-γ-AApeptides to mimic long helical hormone peptide GLP-1, and showed that certain sequences could reproduce the functionality of GLP-1 on multiple helical faces and effectively activated glucose uptake upon binding to GLP-1R, which could be further developed to treat type 2 diabetes.

1.4 The application of macrocyclic γ-AApeptides

High-throughput screening (HTS) is a well-established strategy for identifying conventional drug targets. As a result, our group developed a one-bead-two-compound library to screen against PPIs interface hotspots to find potential inhibitors. Using this macrocyclic library, Yan and her coworkers were able to identify a potent ligand in 2017 that specifically binds to the
EphA2 receptor tyrosine kinase with a Kd value of 81 nM and inhibits EphA2 signaling in both \textit{in vitro} and cellular assays. Then, using the same library to screen against the extracellular domain of the EGFR, our research group discovered a molecule.\textsuperscript{16} This molecule could also effectively antagonize EGF-stimulated EGFR phosphorylation and downstream signal transduction, which may be further explored as an anti-cancer medication.\textsuperscript{16} We recently discovered a macrocyclic peptide that can tightly bind with E6AP HECT domain by this method, accelerating the degradation of E6AP substrates in the cell by increasing E6AP's enzymatic activities of E6AP.\textsuperscript{17} This peptide could be developed alongside E3 inhibitors and substrate recruiters such as PROTACs and molecular glues to leverage the full potential of protein ubiquitination pathways for drug development.\textsuperscript{17}

\textbf{Figure 1 The chemical structure of \(\gamma\)-AA peptides and sulfonyl-\(\gamma\)-AA peptides}

(A) Structure of sulfonyl-\(\gamma\)-AA peptide building block. (B) Schematic representation of distribution of side chains from sulfonyl-\(\gamma\)-AA peptides. (C) Crystal structure of a sulfonyl-\(\gamma\)-AA peptide. (D) Top view of (C). Reproduced with the permission of American Chemical Society.\textsuperscript{5}
1.5 Outline of dissertation

In Chapter 2, we used combinatorial library to identify a novel macrocyclic \( \gamma \)-AApeptides-based pan-coronavirus fusion inhibitor by targeting on RBD and HR1 in Spike protein of SARS-CoV-2. This molecule was found to be used as orally antiviral therapeutic and prophylactic candidate against current SARS-CoV-2 and its variants, as well as future emerging and re-emerging HCoVs.

In Chapter 3, using the strategy of rational design, we designed and synthesized a series of right-handed helical mimetics D- sulfonyl-\( \gamma \)-AApeptides that could mimic HR2 and specifically bind to HR1 domain in S protein to inhibit the fusion of SARS-CoV-2. To the best of our knowledge, this is the first peptidomimetics based on unnatural framework to disrupt this protein-protein interaction (PPI).

In Chapter 4, we designed and synthesized helical sulfonyl-\( \gamma \)-AApeptides that can mimic C34 of HIV fusion domain and disrupt 6-helix bundle formation involved in HIV fusion, leading to potent and broad-spectrum anti-HIV regents.

1.6 References


Chapter 2  A Novel Cyclic γ-AApeptide-based Long-acting Pan-coronavirus Fusion Inhibitor with Oral Bioavailability by Targeting Two Sites in Spike Protein

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2.1 Introduction

Several vaccines\(^1\) and therapeutics\(^2\) have been approved for use against COVID-19 caused by SARS-CoV infection. However, their effectiveness against the emerging variants of SARS-CoV-2, such as the B.1.1.7 (Alpha),\(^3\) B.1.351 (Beta),\(^4\) B.1.1.248 (Gamma),\(^5\) B.1.617.2 (Delta),\(^6\) and B.1.1.529 (Omicron)\(^7\) can decline. Therefore, developing more effective and broader spectrum prophylactics and therapeutics is still urgently needed.

Coronaviruses consist of four genera: Alphacoronavirus (α), Betacoronavirus (β), Gammacoronavirus (γ) and Deltacoronavirus (δ).\(^7\) Two Alphacoronaviruses (HCoV-NL63 and HCoV-229E) and 5 Betacoronaviruses, including low pathogenic CoVs (HCoV-OC43, HCoV-HKU1) and 3 high pathogenic CoVs (SARS-CoV, MERS-CoV, and SARS-CoV-2) can infect humans.\(^8,9-12\) To date, several strategies have been adopted for the development of anti-SARS-CoV-2 therapeutics by targeting viral spike (S) protein (S1 and S2 subunits), viral enzymes (PLpro, 3CLpro, RdRp and helicase),\(^13\) and some structure proteins.\(^13\) Generally, small molecular inhibitors with oral bioavailability are more suitable for intracellular targets, *i.e.*, viral proteases,
by the necessity of cell permeability. One inhibitor of main protease (M\(^{\text{pro}}\)/3C-like protease (3CLpro), Paxlovid\textsuperscript{TM}, was recently approved by the US FDA as an oral drug for treatment of SARS-CoV-2 infection.\textsuperscript{14} However, instances of reinfection after completing the recommended course of Paxlovid are reported\textsuperscript{15} and recent study shows that this type of M\(^{\text{pro}}\) inhibitors tends to induce rapid drug resistance.\textsuperscript{16,17}

SARS-CoV-2 neutralizing antibodies (nAbs) generally target RBD in S1 subunit.\textsuperscript{18-21} However, nAbs lack oral bioavailability and lose neutralizing activity against SARS-CoV-2 variants that escape immune surveillance.

Jiang’s group previously identified a series of pan-CoV fusion inhibitors, such as EK1 peptide and EK1C4 lipopeptide, targeting the heptad repeat 1 (HR1) domain in S2 subunit of SARS-CoV-2 S protein with highly potent antiviral activity against all HCoVs tested,\textsuperscript{22,23} demonstrating the potential of using S protein to develop pan-antiviral inhibitors. de Vries et al.\textsuperscript{24} synthesized a dimeric lipopeptide [SARS\(_{\text{HRC}}\)-PEG\(_4\)]\(_2\)-chol, and with daily intranasal administration to SARS-CoV-2 ferrets, it could completely prevent SARS-CoV-2 direct-contact transmission with limited toxicity. Despite providing excellent inhibitory against SARS-CoV-2 virus and broad-spectrum antiviral activity,\textsuperscript{22,23,25} however, these peptides generally suffer from low enzymatic stability and poor oral bioavailability. Therefore, this study aimed to identify peptide-based pan-CoV fusion inhibitors with high proteolytic enzyme stability and good oral bioavailability.

Cai’s group previously established several cyclic \(\gamma\)-AApeptide-based one-bead-two-compound (OBTC) combinatorial libraries in which the cyclic \(\gamma\)-AApeptides possess high proteolytic enzyme stability and good oral availability.\textsuperscript{26-30} Through screening these OBTC libraries, several important hits, such as cyclic \(\gamma\)-AApeptides targeting EphA2, EGFR and HER2
were identified,$^{27,30,31}$ suggesting that these libraries can be used for identification of γ-AApeptide-based pan-CoV fusion inhibitors with oral bioavailability.

![Diagram of library to screen for inhibitors targeting SARS-CoV-2 S protein](image)

**Figure 2 Library to screen for inhibitors targeting SARS-CoV-2 S protein**

(a) Schematic presentation of OBTC TentalGel beads. Chemical structures of γ-A peptide (i) and α-peptide (ii). (b) Scheme showing the overall strategy.

Here, we (Jiang’s and Cai’s groups) worked together to screen a cyclic γ-AA peptide-based OBTC combinatorial library against SARS-CoV-2 spike protein, and after the first screening, we identified $\text{S-20}$, a hit with potent fusion and entry inhibitory activity, but moderate selectivity index (SI). Through the modification of the hit, we found that the analog compound, $\text{S-20-1}$, exhibited potent fusion and entry inhibitory activity against SARS-CoV-2 and its variants as well as other HCoVs, such as HCoV-OC43 and had exceptionally high SI. $\text{S-20-1}$ also demonstrated excellent *in vivo* efficacy by potently inhibiting both HCoV-OC43 and SARS-CoV-2 infections in mice and
good *in vivo* safety profiles. Most importantly, S-20-1 was highly resistant to proteolytic degradation and exhibited favorable oral bioavailability, suggesting a great potential to be further developed as a therapeutic and prophylactic for treatment and prevention of infection by SARS-CoV-2 and its variants as well as other HCoVs.

![Figure 3 Screening for SARS-CoV-2 fusion and entry inhibitors from a cyclic γ-AApeptide library](image)

Inhibition of cell-cell fusion mediated by the S protein of SARS-CoV-2 by putative hits at 50 μM (a) and 5 μM (b). (c) Chemical structures of seven hits. (d) Inhibitory activity of hits from SARS-CoV-2 pseudovirus infection assay. (e) Cytotoxicity of hits.
2.2 Results

2.2.1 Library design, synthesis, and screening

Inspired by the backbone of the chiral peptide nucleic acid (PNA), we recently developed a class of peptidomimetic γ-AApeptides which shows remarkable resistance to proteolytic degradation, robust helical folding propensity, and promising applications in biomedical sciences. The chemodiversity and modular synthesis of γ-AApeptides make them ideal candidates to create combinatorial libraries bearing unnatural ligands. To date, macrocyclic γ-AApeptides have been identified to bind nucleic acids and proteins with high affinity and specificity. Here, we screen a library of γ-AApeptides against S protein of SARS-CoV-2.

We first constructed an OBTC combinatorial library comprised of thioether-bridge-mediated cyclic γ-AApeptides as reported previously (Figure 2). They contained a diverse and random set of hydrophobic and charged side chain units, resulting in a theoretical diversity of 320,000 compounds, with each compound being encoded by an 8-mer peptide (Figure 2a). The OBTC library was incubated with His-tag SARS-CoV-2 S protein, followed by incubation with Dylight 488 6x-His Tag Monoclonal Antibody (Figure 2b). Putative positive beads were microscopically identified from the library pool, and the encoding peptides were analyzed by tandem MS/MS of MALDI. The chemical structures of 43 putative hits were determined unambiguously.
Figure 4 Identification of four modified cyclic γ-AApeptides with improved SARS-CoV-2 fusion/entry inhibitory activity and SI

(a) Chemical structures of four modified hits. (b) HeLa cells incubated with FITC labeled S-20 (i-iii) and FITC labeled S-20-1 (iv-vi) at 1 μM for 2 h, respectively, and then stained with DAPI. (i) DAPI channel; (ii) FITC channel; (iii) merged. (iv) DAPI channel; (v) FITC channel; (vi) merged. (c) Inhibitory activity of 4 modified cyclic γ-AApeptides in PsV infection assays against SARS-CoV-2. (d) Cytotoxicity of 4 modified cyclic γ-AApeptides in Huh-7 cell line. (e) Inhibitory activity of S-20-1 on authentic SARS-CoV-2 replication in Caco-2 cell line.

2.2.2 S-20-1, a modified cyclic γ-AApeptide, exhibited high fusion inhibitory activity and low cytotoxicity

We first assessed the inhibitory activity of these 43 putative hits in vitro using a SARS-CoV-2 S protein-mediated cell-cell fusion assay established in our lab. Under 50 μM
concentration, 29 hits exhibited more than 50% inhibition (Figure 3a). To confirm inhibitory activity and select final hits for further investigation, these compounds were tested again at a 5 μM concentration (Figure 3b), revealing seven compounds (S-13, S-20, S-23, S-24, S-25, S-30, and S-32) still able to efficiently inhibit SARS-CoV-2 S-mediated cell-cell fusion (> 80%). To further validate these compounds (Figure 3c), we used our well-established SARS-CoV-2 pseudovirus (PsV) infection assay to assess their inhibitory activity of these compounds against SARS-CoV-2 PsV infection as indicated by the half maximal inhibitory concentration (IC$_{50}$). Their cytotoxicity was simultaneously evaluated, and half-maximal cytotoxic concentration (CC$_{50}$) was calculated to determine selectivity index (SI=CC$_{50}$/IC$_{50}$). All seven compounds showed excellent antiviral activities against SARS-CoV-2 PsV infection with IC$_{50}$ of 1-5 μM (Figure 3d, Table 1). These compounds only exhibited cytotoxicity at noticeably higher concentration (Figure 3e) and revealed decent selectivity with SI between 1.6 and 14.3 (Table 1).

We next asked if SI of these hits could be further improved. We speculated that the low-to-moderate SI was caused by the ability of cyclic γ-AApeptides to cross the host cell membrane and potentially work on intracellular targets. Therefore, negative charges could be introduced to decrease cell permeability, thereby minimizing potential cytotoxicity and increasing SI. We then added two negative charges to each of four compounds S-20, S-23, S-24, S-25 (Figure 4a) that showed the best PsV inhibitory activity, and their ability to cross the cell membrane declined. No fluorescence was observed for S-20-1 at 1 μM (Figure 4b v) after incubation with HeLa cells compared to S-20 (Figure 4b ii) at the same condition, which showed strong fluorescence, demonstrating the abolishment of cell permeability of S-20-1.

The modified compounds were then tested for antiviral activity and cytotoxicity using the PsV assay (Figure 4c) and cytotoxicity assay (Figure 4d). As shown in Table 2, modification of
the compounds with negative charges did not significantly alter their antiviral activity. **S-20-1** even revealed a >3-fold better activity (IC\(_{50}\): 0.8 μM) compared with **S-20** (IC\(_{50}\): 2.9 μM), suggesting no effect of modification on the binding of these cyclic peptidomimetics toward S protein. Cytotoxicity of these compounds (Figure 4d) was also largely diminished, leading to a remarkable improvement of SI (95 ~ >1,000) (Table 2). With IC\(_{50}\) of 0.8 μM and the CC\(_{50}\) of more than 800 μM, **S-20-1** exhibited an exceptional SI (>1,000). Based on its performance, we selected **S-20-1** and tested its inhibitory activity against authentic SARS-CoV-2 infection of Caco-2 cells. As anticipated, **S-20-1** effectively blocked authentic SARS-CoV-2 infection at the cellular level in a dose-dependent manner with an IC\(_{50}\) of 8.14 μM. (Figure 4e), consistent with the results from the PsV infection assay. Taken together, **S-20-1** was demonstrated to be a potent and highly selective inhibitor against SARS-CoV-2 infection (Figure 4e, Table 2).

### 2.2.3 **S-20-1 efficiently inhibited various SARS-CoV-2 variants in different cell lines**

Next, we evaluated the *in vitro* efficacy of **S-20-1** against infection by SARS-CoV-2 variants, as cited previously, and on different cell lines. We found that **S-20-1** potently inhibited infection by pseudotyped B.1.1.7 (Figure 5a), B.1.351 (Figure 5b), P.1 (Figure 5c), C.37 (Figure 5d), B.1.617.2 (Figure 5e), B.1.1.529 (Figure 5f), and the mutant with N501Y, K417N and E484K mutations (Figure 5g) in the Huh-7 cell line with IC\(_{50}\) values ranging from 0.54 to 10.23 μM. We also tested the anti-PsV activity of **S-20-1** against some of the most virulent SARS-CoV-2 variants in Caco-2 cells. Its inhibitory activity was consistent with that for Huh-7 cells, revealing IC\(_{50}\) values ranging from 4.44 to 6.37 against B.1.1.7 (Figure 5h), B.1.351 (Figure 5i), B.1.617.2 (Figure 5j) and B.1.1.529 (Figure 5k). Together, **S-20-1** exhibited broad-spectrum inhibitory activity against predominant SARS-CoV-2 variants.
Figure 5 Inhibition of S-20-1 against infection by pseudotyped SARS-CoV-2 variants on different cell lines

Inhibition of infection by PsV of SARS-variants on Huh-7 cells: (a) B.1.1.7 (Alpha), (b) B.1.351 (Beta), (c) P.1 (Gamma), (d) C.37 (Lambda), (e) B.1.617.2 (Delta), (f) B.1.1.529 (Omicron), (g) mutant with N501Y, K417N, and E484K mutation. Inhibition of infection by PsV of SARS-variants on Caco-2 cells: (h) B.1.1.7 (Alpha), (i) B.1.351 (Beta), (j) B.1.617.2 (Delta), and (k) B.1.1.529 (Omicron).

2.2.4 **S-20-1 potently inhibited cell-cell fusion-mediated by S proteins of 5 HCoVs and blocked infection by 4 pseudotyped HCoVs and 1 pseudotyped bat SARSr-CoV, as well as 2 authentic HCoVs**

As an analog of S-20, S-20-1 was expected to have strong binding affinity to S protein and have broad-spectrum antiviral activity against diverse HCoVs, including α-HCoV and β-HCoV,
since these HCoV share conserved regions in S protein. First, we found that S-20-1 potently inhibited cell-cell fusion mediated by S protein of SARS-CoV-2 (Figure 6a), SARS-CoV (Figure 6b), MERS-CoV (Figure 6c), HCoV-229E (Figure 6d) and HCoV-NL63 (Figure 6e) with IC50s ranging from 1.47 to 5.44 μM, confirming that S-20-1 is a pan-HCoV fusion inhibitor. S-20-1 also exhibited potent inhibitory activity against infection of pseudotyped SARS-CoV (Figure 6f), MERS-CoV (Figure 6g), HCoV-229E (Figure 6h), HCoV-NL63 (Figure 6i), and bat SARSr-CoV WIV1 (Figure 6j) with IC50s ranging from 1.30 to 12.02 μM, consistent with the result of SARS-CoV-2 PsV (Figure 4c), indicating that S-20-1 is a pan-CoV entry inhibitor. Finally, like authentic SARS-CoV-2 (Figure 4e), authentic HCoV-OC43 and HCoV-229E infection in RD cells and Huh-7 cells was effectively inhibited by S-20-1 with IC50s of 6.25 μM (Figure 6k) and 9.46 μM (Figure 6l), respectively. Overall, S-20-1 demonstrates broad-spectrum antiviral activity against infection by HCoVs and SARSr-CoVs tested.

2.2.5 Intranasally applied S-20-1 efficiently protected mice from infection by HCoV-OC43 and SARS-CoV-2 Delta variant

To evaluate the protective effect of S-20-1 in vivo, we first used an HCoV-OC43-infected mouse model to assess the prophylactic and therapeutic potential of S-20-1 as an antiviral agent (Figure 7a). S-20-1 was administered to newborn mice in prevention or treatment group via the intranasal route at a single dose of 80 mg/kg 0.5 h pre- or post-challenge with HCoV-OC43 at 100 TCID50, respectively. At four days post-infection, mice were sacrificed, and brains excised to evaluate viral load. As shown in Figure 7b, relative HCoV-OC43 RNA level of both prevention and treatment group was significantly lower than that of non-treatment control group. Results showed that S-20-1 could effectively protect newborn mice from infection of HCoV-OC43.
Figure 6 Inhibition of S-20-1 on infection of divergent HCoVs and SARSr-CoV

Inhibitory activity of S-20-1 on cell-cell fusion mediated by the S protein of SARS-CoV-2 (a), SARS-CoV (b), MERS-CoV (c), 229E (d), and NL63 (e). Inhibitory activity of S-20-1 on infection of pseudotyped SARS-CoV (f), MERS-CoV (g), 229E (h), NL63 (i) and SARs-CoV WIV1 (j). Inhibitory activity of S-20-1 on infection of authentic HCoV-OC43 (k) and HCoV-229E (l).

We then tested the protective efficacy of S-20-1 on SARS-CoV-2 Delta variant-infected hACE2-transgenic mouse model, C57BL/6-Tgtn (CAG-human ACE2-IRES-LuciferaseWPRE-polyA) as described before (Figure 7c). First, we assessed inhibitory activity against the SARS-
CoV-2 Delta variant on Caco-2 cells in vitro, and viral load was significantly decreased about 2 logs (100-fold) at 50 µM concentration of S-20-1 (Figure 7d). Then we intranasally administered S-20-1 at the dose of 60 mg/kg to hACE2 transgenic mice (female, eight weeks old) 0.5h before (prevention group) or after (treatment group) at 10,000 pfu of SARS-CoV-2 Delta variant via the intranasal route. The viral load in the brain of mice in the prevention and treatment groups was 2.02 and 2.16 logs (~100-fold), respectively, compared to that in the non-treatment control group (Figure 7e). In the lung of mice in the prevention and treatment groups, it was 2.5 logs (~300 fold) and 2.3 logs (~200 fold), respectively, lower than that in the non-treatment control group (Figure 7f). Therefore, intranasally administered S-20-1 exhibited prophylactic and therapeutic effect against SARS-CoV-2 Delta infection.

2.2.6 S-20-1 inhibited SARS-CoV-2 infection at the early stage of viral entry

To gain mechanistic insight of S-20-1 against SARS-CoV-2 infection, we first used the time-of-removal assay to determine whether the inhibitory activity of S-20-1 resulted from binding to virus or host cell surface to block SARS-CoV-2 entry. We incubated S-20-1 with Huh-7 cells at 37 °C for 1 h and then washed cells with PBS before SARS-CoV-2 was added. No inhibitory activity was observed after washing (Figure 8a), suggesting that S-20-1 targets viruses, not host cells. Next, S-20-1 was added to Huh-7 cells at different time points before, during, and after SARS-CoV-2 infection to determine the affected stage of the viral life cycle. As shown in Figure 8b, S-20-1 exhibited more than 80% inhibition of SARS-CoV-2 infection when added 0.5 h before, at the same time (0 h), or 0.5 and 1 h after the addition of virus. The inhibitory activity was then gradually decreased to ~60% at 2 and 4 h, 35% at 6 h and 15% at 8 h, indicating that S-20-1 may target the early stage of the virus life cycle. Next, we used a previously reported assay by adjusting
the temperature to distinguish the process of entry, post-entry, attachment, and post-attachment.\textsuperscript{38} As shown in Figure 8c, \textbf{S-20-1} could inhibit 80\%, 70\% and 75\% in the entry stage, attachment stage and post-attachment stage, respectively, with no effect at the post-entry stage, in good agreement with its targeting at the early fusion stage. We also assessed the inhibitory activity of \textbf{S-20-1} against HCoV-OC43 infection with the same assays described above. As shown in Figure 8d and 8e, \textbf{S-20-1} exhibited results similar to those when SARS-CoV-2 was tested, suggesting that \textbf{S-20-1} targets the early entry stage of SARS-CoV-2, HCoV-OC43, and possibly other HCoVs.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{Prevention and treatment effect of \textbf{S-20-1} against mouse infection by HCoV-OC43 and SARS-CoV-2 Delta variant}
\end{figure}

(a) Schematic diagram of \textbf{S-20-1} administration and HCoV-OC43 challenge. (b) \textit{In vivo} efficacy of \textbf{S-20-1} (80 mg/kg) against HCoV-OC43 infection in newborn mice. Viral RNA expression level in brain tissue of mice in each group on the 4th day post-infection was detected. (c) Schematic diagram of \textbf{S-20-1} administration and SARS-CoV-2 challenge. (d) Viral RNA expression level after incubation of \textbf{S-20-1} with authentic SARS-CoV-2 Delta on Caco-2 cells. (e, f) \textit{In vivo} efficacy of \textbf{S-20-1} (60 mg/kg) against SARS-CoV-2 Delta variant infection in hACE2-transgenic C57BL/6 mice. Viral RNA expression level in mouse brain (e) and lung (f) of each group on the 4th day post-infection was detected. These data were analyzed by Student’s \textit{t} test (d) and One-way ANOVA (b, e, f).
Figure 8 Inhibition of SARS-CoV-2 infection by S-20-1 at the early stage of viral entry

(a) In PsV infection assay, Huh-7 cells were pretreated with S-20-1 at 50 µM at 37 °C for 1 h, washed with PBS to remove unbound S-20-1, and infected with SARS-CoV-2 at 37 °C. (b, d) In time-of-addition assays, Huh-7 and RD cells were treated with S-20-1 at the indicated time points before or after addition of pseudotyped SARS-CoV-2 (b) and authentic HCoV-OC43 (d), respectively. Supernatants containing free S-20-1 and viral particles were removed 12 h later. (d, e) A series of well-established assays were performed to confirm the stage at which S-20-1 blocked entry of SARS-CoV-2 or HCoV-OC43 into target cells. Data were analyzed with One-way ANOVA (A) and Two-way ANOVA (c, e). NS means no significance.
The binding affinity of S-20 and S-20-1 toward various subunits in S protein was then
determined by fluorescence polarization assays.33 Both compounds exhibited excellent binding
affinity toward S1 subunit with Kd of 50 nM (Figure 9a) and 67 nM (Figure 9b), respectively. This
similarity confirmed that modification of S-20 with negative charges did not change its binding
activity to S protein. We next measured the binding affinity of both compounds toward RBD,
revealing Kd values of 57 nM (Figure 9c) and 61 nM (Figure 9d), respectively, suggesting that
both S-20 and S-20-1 mainly target RBD on S1 subunit, which may account for their excellent
inhibitory activity against SARS-CoV-2 in vitro. However, potent binding affinity to S1 subunit
alone could not plausibly explain why S-20-1 exhibited broad-spectrum antiviral activity against
various HCoVs, as the S1 subunit is not well conserved in S protein. Recalling that Jiang’s group
had previously identified EK1 peptide and EK1C4 lipopeptide targeting the HR1 domain in S2
subunit of SARS-CoV-2 S protein,22,23,25 we speculated that S-20-1 might also bind to this domain.
Indeed, we found that S-20 and S-20-1 bound with HR1 domain tightly with Kd values of 92 nM
(Figure 9e) and 277 nM (Figure 9f), respectively, possibly explaining the broad-spectrum activity
of S-20-1 toward various HCoVs. Interestingly, neither S-20 nor S-20-1 bound to the HR2 domain,
which is consistent with results from pan-CoV fusion inhibitor EK1 peptide that binds with HR1,
but not HR2. These results suggest that S-20-1 inhibits SARS-CoV-2 infection, possibly by
binding RBD in S1 subunit and HR1 region in S2 subunit of S protein on virus separately. Co-
crystallographic analysis of the S-20-1/S protein complex should be performed in the future, in
order to determine whether or not S-20-1 can bind RBD and HR1 regions in one S protein
simultaneously.
Figure 9 Binding of S-20-1 or S-20 with S1, RBD, or HR1, respectively

Affinity of binding between S-20 and S1 (a), S-20-1 and S1 (b), S-20 and RBD (c), S-20-1 and RBD (d), S-20 and HR1, or S-20-1 and HR1 (f).

2.2.7 Docking of S-20, which shares the active group of S-20-1, with RBD or HR1

Using the Schrödinger Glide docking program, we could not dock S-20-1 with either RBD or HR1 because of S-20-1’s long tail. When we reperformed the docking analysis with S-20, which has no tail, to mimic S-20-1, we found that S-20 could bind with both RBD and HR1 via a number of hydrophilic and hydrophobic interactions.

As shown in Figure 10a and 10b, the hydrophobic side chains of 1a, 2a, 2b, 3b, and 4a of S-20 could form either π-π interaction or hydrophobic interactions with either one or more residues of 473Y, 421Y, 455L, 456F, 489Y and 486F on RBD owing to close contacts. In addition, cationic 1b and hydrophilic 4b may form hydrogen bonding with 459S and 487N, respectively. On
the other hand, the arrangement of the hydrophobic and hydrophilic groups on S-20 also enables its favorable binding with the residues on HR1 (Figure 10c and 10d). For instance, 1a of S-20 deeply inserted into the hydrophobic domain formed by 930A, 931I, 934I, and 938L from three HR1 chains on the HR1/HR2 fusion core. Additionally, 2b, 3b and 4a potentially adopted hydrophobic contacts with 942A, 945L and 944A across different HR1 chains. Hydrophilic interactions between S-20 and HR1 could also be identified, including potential hydrogen bonds forming between 1b and 935Q, 4b and 936D, respectively. Most residues in HR1 are highly conserved among HCoVs, including SARS-CoV-2, SARS-CoV, MERS-CoV, HCoV-229E, and HCoV-NL63/OC43, which may explain why S-20 and S-20-1 have broad-spectrum inhibitory activity against divergent HCoVs like EK1 peptide. Overall, computational simulation is highly consistent with experimental data, potentially providing guidance to optimize and develop more potent derivatives.

2.2.8 S-20-1 was resistant to various proteolytic enzymes in blood

We next assessed the metabolic stability of S-20-1 in the presence of proteinase K and trypsin. As shown in Figure 11a and 11b, the inhibition of SARS-CoV-2 PsV infection showed no decrease within 4 hours’ incubation of S-20-1 in the presence of proteinase K and trypsin. Next, S-20-1 was incubated for 24 h with Pronase, a broad-specificity mixture of proteases extracted from Streptomyces griseus, followed by analysis with RP-HPLC. S-20-1 was remarkably stable and showed no noticeable degradation, even at 24 h (Figure 11c and 11d), indicating its high resistance to various proteolytic enzymes in blood.
Molecular docking analysis of the interaction between S-20, which shares the active group in S-20-1, and RBD (a and b) or HR1 (c and d). Side chains of S-20 are designated by a (chiral side chain) or b (acyl side chain) in each AAPeptide building block, respectively. Residues of HR1 from different helical chains are shown in red, black, and purple, respectively.
2.2.9 *S*-20-1 possessed favorable passive permeability to the blood brain barrier (BBB) and gastrointestinal tract (GIT) membranes, suggesting good oral bioavailability

The parallel artificial membrane permeability assay (PAMPA) is a high-throughput screening (HTS) technique to predict passive permeability by numerous different biological membranes, such as the gastrointestinal tract (GIT), blood brain barrier (BBB), and dermal layer.$^{39}$ Here, we employed PAMPA-BBB to evaluate the ability of *S*-20-1 to penetrate the BBB and PAMPA-GIT to determine the gastrointestinal absorption rate and thus predict the oral bioavailability of *S*-20-1. For the PAMPA-BBB assay, we used Verapamil as positive control and Theophylline as negative control. The $P_{\text{app}}$ values for favorable, medium, and low permeabilities are expected to be $> 20 \times 10^{-6}$ cm/s, $1 \sim 20 \times 10^{-6}$ cm/s and $< 1 \times 10^{-6}$ cm/s, respectively. Surprisingly, *S*-20 and *S*-20-1 at 100 μM displayed favorable permeability with $P_{\text{app}}$ values of 536 $\times 10^{-6}$ cm/s and 30 $\times 10^{-6}$ cm/s, respectively, while Verapamil (positive control) at 50 μM and Theophylline (negative control) at 250 μM exhibited $P_{\text{app}}$ values of 155 $\times 10^{-6}$ and $< 10 \times 10^{-6}$ cm/s, respectively (Figure 12a). These results suggest that both *S*-20 and *S*-20-1 can effectively pass-through BBB, which may explain why *S*-20-1 showed strong protection against SARS-CoV-2 infection in mouse brain.

For the PAMPA-GIT assay, we used Carbamazepine and Antipyrine as the positive and negative controls, respectively, as Carbamazepine is fully orally bioavailable with favorable permeability at pH 5.0, 6.2 and 7.4 with $P_{\text{app}}$ values of 135 $\times 10^{-6}$ cm/s, 158 $\times 10^{-6}$ cm/s and 160 $\times 10^{-6}$ cm/s, respectively, while Antipyrine is poorly orally bioavailable with low $P_{\text{app}}$ values at different pH values. *S*-20-1 displayed favorable permeability at pH 5.0, 6.2 and 7.4 with $P_{\text{app}}$ at 616 $\times 10^{-6}$ cm/s, 326 $\times 10^{-6}$ cm/s and 31 $\times 10^{-6}$ cm/s, respectively (Figure 12b). These results
suggest that S-20-1 may have a higher absorption rate under fed conditions than that in fasted conditions. Therefore, S-20-1 is expected to have potential oral bioavailability.

Figure 11 Evaluation of the resistance of various proteolytic enzymes

Metabolic stability of S-20-1 in proteinase K (a) and trypsin (b). Analytic HPLC traces of S-20-1 before (c) and after (d) incubation with Pronase (0.1 mg/ml).
2.2.10 S-20-1 exhibited excellent pharmacokinetic (PK) profile and oral bioavailability tested in mouse model

To exploit the in vivo stability and oral bioavailability of S-20-1, we investigated its pharmacokinetics (Figure 12c) by administering S-20-1 in mice via intraperitoneal (IP) and oral
administration (PO) of \textbf{S-20-1} at 50 mg/kg over 48 h, respectively. For IP administration, \textbf{S-20-1} demonstrated excellent PK parameters with a long half-life (t_{1/2}) of 14.53 h and a high peak concentration (C_{max}) of 120,637 µg/L (Figure 12d). For PO administration, \textbf{S-20-1} exhibited an even longer half-life (t_{1/2} = 24.29 h) and an excellent oral bioavailability of ~2.4%, compared to IP.

### 2.2.11 \textit{S-20-1 had good in vivo safety profiles in mouse model}

Eight-week-old Balb/c mice were used to test the \textit{in vivo} safety of \textbf{S-20-1}. Mice were administered with \textbf{S-20-1} intranasally once daily for three days, and their bodyweight was monitored every day for 12 days (Figure 13a). The bodyweight of mice in both \textbf{S-20-1} and PBS groups exhibited no significant changes (Figure 13b). We then euthanized the mice on the 12th day (Figure 13a) and collected their liver, lung, kidney, and brain tissues. Histological sections of the tissues were stained with HE and examined microscopically. Both \textbf{S-20-1} and PBS groups showed similar histological features (Figure 13c). No inflammatory changes were observed in these tissues, suggesting that \textbf{S-20-1} is safe.

### 2.3 Discussion

SARS-CoV-2 S protein consists of several important targets for the development of viral fusion and entry inhibitors.\textsuperscript{32} For example, neutralizing antibodies (nAbs) and other proteins inhibit SARS-CoV-2 infection by binding RBD in S1 subunit and blocking viral attachment to the receptor on the host cell.\textsuperscript{18-21} Peptides derived from the HR2 domain, such as 2019-nCoV-HR2P, suppress SARS-CoV-2 fusion and entry by interacting with the HR1 in S2 subunit and interfering with the interaction between HR1 and HR2 to form the fusion-active 6-HB.\textsuperscript{40}
Figure 13 *In vivo* safety evaluation of S-20-1

(a) Flow diagram of *in vivo* safety experiments. (b) Bodyweight changes of mice administered with S-20-1 or PBS intranasally. (c) Histological changes of mouse lung, liver, brain, and kidney after administration with S-20-1 or PBS intranasally. Tissues were stained with HE. The scale bar shown in slides were 1,000 µm and 100 µm, respectively.

Jiang’s group previously identified a series of pan-CoV fusion inhibitors, such as EK1 peptide and EK1C4 lipopeptide, targeting the HR1 domain in S2 subunit of SARS-CoV-2 S protein with highly potent antiviral activity against all HCoVs tested.\(^{22,23}\) Therefore, these peptide-based pan-CoV fusion inhibitors can be developed for intranasally applied therapeutics for treatment of SARS-CoV-2 infection.\(^{37}\) However, their future clinical use may not be preferably selected
because of their lack of oral bioavailability. Meanwhile, Cai’s group previously established several cyclic γ-AApeptide-based OBTC combinatorial libraries in which the cyclic γ-AApeptides possess high proteolytic enzyme stability and potent biological activity.26-30 For example, several cyclic γ-AApeptides were identified to target EphA2, EGFR and HER2 with excellent binding affinity and specificity.27,30,31 Therefore, it is feasible to identify some γ-AApeptide-based pan-CoV fusion inhibitors with oral bioavailability.

Here, Jiang’s and Cai’s groups worked together to identify cyclic γ-AApeptide-based pan-CoV fusion and entry inhibitors with oral bioavailability. More specifically, a cyclic γ-AApeptide-based OBTC combinatorial library was first screened against SARS-CoV-2 S protein and 43 active beads with SARS-CoV-2 S protein-mediated cell-cell fusion inhibitory activity at 50 μM were identified. Upon validation, seven potential hits were selected for further evaluation using SARS-CoV-2 PsV infection assay. The four best hits with better PsV inhibitory activity, including S-20, were selected for modification. We found that one of the derivative compounds, S-20-1, exhibited the most potent inhibitory activities against infection by pseudotyped and authentic SARS-CoV-2 and highest SI (>1,000).

Most importantly, S-20-1 is highly resistant to proteolytic degradation (showing no noticeable degradation up to 24 h when it was incubated with Pronase) and has a long half-life (~24 h) with oral administration, which is much longer than the that (~2 h) of nirmatrelvir through oral administration.41 We believe that the following two reasons may explain why S-20-1 with small size has a long half-life: 1) the unnatural backbones in γ-AApeptides are highly resistant to enzymatic hydrolysis, and 2) the cyclization of γ-AApeptides can rigidify functional groups to further increase stability towards proteolysis. In addition, S-20-1 possesses favorable oral bioavailability with P_{app} values of 30 × 10^{-6} cm/s. To further confirm its proteolytic stability, long
half-life, and oral bioavailability, we will perform experiments to evaluate the prophylactic and therapeutic effects through the oral route once daily in the future. If these are confirmed, \textbf{S-20-1} has the potential to be used in combination therapies with other orally applicable COVID-19 drugs with different mechanism of action or targeting different proteins, such as $M^{pro}$ inhibitors (e.g., Paxlovid).\textsuperscript{42} These combinations may have synergistic effect and raise the genetic barrier to drug resistance.

Mechanistic studies suggested that \textbf{S-20-1} acts at the early entry stage of the viral life cycle, including attachment, post-attachment stages and fusion stage, but not the post-entry stage. Further investigation demonstrates that \textbf{S-20-1} has dual targets in S protein, including RBD in the S1 subunit and HR1 in S2 subunit, suggesting that it inhibited SARS-CoV-2 fusion with and entry into the host cell by binding with RBD to block its interaction with the ACE2 receptor on the host cell, just like neutralizing antibodies, and interacting with HR1 to interfere with fusion activity and 6-HB formation, just like EK1 (Figure 14). Of course, it is impossible to allow one cyclic peptide to bind both RBD and HR1 at the same time because of its limit size. We believe that different S-\textbf{20-1} molecules may bind RBD and HR1 simultaneously or separately to inhibit viral infection. HR1 is a highly conserved domain in S protein of HCoVs, providing the basis of broad-spectrum anti-HCoV activity of \textbf{S-20-1} like the peptide-based pan-CoV fusion inhibitors EK1 and EK1C4.\textsuperscript{22,23} Moreover, as \textbf{S-20-1} could potently bind with RBD and HR1 in the spike protein, it is expected to be hard to generate the drug resistance in the clinical application.

\textbf{2.4 Conclusion}

Based on our previous experience in developing peptide-based pan-CoV fusion inhibitors and cyclic $\gamma$-AApeptide-based protein binders, we herein identified a modified cyclic $\gamma$-
AApeptide-based pan-CoV fusion and entry inhibitor, **S-20-1**. By targeting the RBD in S1 subunit and HR1 in S2 subunit of S protein, **S-20-1** exhibited potent and broad-spectrum inhibitory activity against infection by SARS-CoV-2, its variants, and other HCoVs, as well as bat SARSr-CoVs. It protected mice from infection of SARS-CoV-2 and HCoV-OC43 infection with a good *in vivo* safety profile. Most importantly, **S-20-1** was highly resistant to proteolytic degradation, and it exhibited long half-life and favorable oral bioavailability. These results suggested that **S-20-1** is a promising orally deliverable antiviral therapeutic and prophylactic candidate against current SARS-CoV-2 and its variants, as well as future emerging and re-emerging HCoVs.

**Figure 14 Proposed mechanism of action of S-20-1 against SARS-CoV-2 infection**

The entry of SARS-CoV-2 into the host cell is initiated by binding of RBD in S1 subunit of S protein to ACE2 (the receptor of SARS-CoV-2), which triggers the conformation change of S2 subunit of S protein and exposes the fusion intermediate structure consisting of HR1, HR2, and fusion peptide (FP). Then, HR1 and HR2 interact with each other to form 6-HB, bringing the viral and host cell membranes together for fusion. Like SARS-CoV-2 nAb and EK1 peptide, **S-20-1** is able to bind with RBD in S1 subunit and HR1 in S2 subunit to block viral attachment and fusion, respectively. Different from nAb and EK1 peptide, **S-20-1** also has oral bioavailability like Paxlovid™, noted above, which targets the intracellular main protease (Mpro). The figure was created with BioRender.com. However, **S-20-1** is superior to peptide- and lipopeptide-based pan-CoV fusion inhibitors because it is much more resistant to proteolytic enzymes and has a longer half-life than EK1, as well as good oral bioavailability. Therefore, **S-20-1** has better potential to be developed as an orally usable drug for treatment of SARS-CoV infection.
2.5 Materials and methods

2.5.1 Materials

All chemicals were purchased from commercial suppliers and directly used without further purification. Fmoc-protected amino acids were purchased from Chem-impex and used for the building block preparation. TentaGel resin (0.23 mmol/g) used for OBTC library preparation was purchased from RAPP Polymere. Rink Amide-MBHA resin (0.55 mmol/g) used for the synthesis of cyclic γ-AA peptides was purchased from GL Biochem. Analysis and purification of cyclic γ-AA peptides was performed on the Waters Breeze 2 HPLC system and lyophilized on a Labcono lyophilizer. Purity of the compounds was determined to be > 95% by analytical HPLC. The mass of each compound was confirmed by high-resolution mass spectrometry detected by Agilent 6220 using electrospray ionization time-of-flight (ESI-TOF). MS/MS analysis for the decoding sequence was obtained with an Applied Biosystems 4700 Proteomics Analyzer.

293T, RD, and Caco-2 cells were purchased from ATCC and stocked in our laboratory. Huh-7 cells were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China). Caco-2 cells were cultured in MEM containing 10% FBS. Other cells were cultured with DMEM containing 10% FBS. HCoV-OC43 (VR-1558) and HCoV-229E (VR-740) were obtained from ATCC and propagated in our laboratory. SARS-CoV-2 (nCoV-SH01, GenBank number: MT121215.1) and SARS-CoV-2 Delta variant were isolated by Fudan University.

2.5.2 One-bead-two-compound library synthesis, screening, and analysis

2.5.2.1 Synthesis

The OBTC library was synthesized following our previous report\textsuperscript{26-30} and the figure for library synthesis is provided below.
2.5.2.2 Prescreening

All TentaGel beads were left to swell in DMF for 1 h, washed with Tris buffer three times, and equilibrated in Tris buffer overnight. After that, the beads were incubated with blocking buffer (1% BSA in Tris buffer with 1000× excess of Escherichia coli lysate) for 1 h. After thorough
washing with Tris buffer, beads were incubated with 6×-His-Tag Monoclonal Antibody (HIS. H8) and Dylight 488 (1: 1000 dilution) for 2 h at room temperature. Beads were washed with Tris buffer, and any beads emitting green fluorescence were picked up manually under microscopy and excluded from the next screening. The remaining beads were washed with Tris buffer and denatured by 8 M guanidine·HCl for 1 h, followed by washing with DI water (5×), Tris buffer (5×) and DMF (5×). Finally, beads were incubated with DMF for 1 h and then equilibrated with Tris buffer overnight.

2.5.2.3 Screening

Beads were incubated with blocking buffer (1% BSA in Tris buffer with 1000× excess of Escherichia coli lysate) for 1 h at room temperature. After washing with Tris buffer four times, beads were incubated with SARS-CoV-2 Spike Protein S1/S2 (aa11-1208) and His Tag Recombinant Protein at the concentration of 50 nM for 4 h with 1% BSA in Tris buffer and 1000× excess of Escherichia coli lysate. After thoroughly washing with Tris buffer, beads were incubated with 6×-His Tag Monoclonal Antibody (HIS. H8) and Dylight 488 (1: 1000 dilution) for 2 h at room temperature. Next, beads were washed with Tris buffer four times and transferred into a six-well plate to be screened under a fluorescence microscope. Beads emitting green fluorescence were picked up as the putative hits.

2.5.2.4 Cleavage and analysis

Each positive bead was transferred into a 1.5 ml Eppendorf microtube and denatured in 100 µL 8 M guanidine·HCl for 1 h at room temperature. After thoroughly washing with Tris buffer, water, DMF, ACN, in the end, the bead was placed into ACN overnight in each microtube and
allowed ACN to evaporate. Beads were cleaved in a 5:4:1 (v/v/v) solution of ACN/glacial acetic acid/H₂O containing cyanogen bromide (CNBr) at a concentration of 50 mg/mL overnight at room temperature. After evaporation, the residue was dissolved in ACN/H₂O (1:1) and analyzed by MALDI-TOF.

2.5.3 Cell permeability assay

The cell permeability study was conducted following our previous report. Briefly, HeLa cells were plated in confocal dishes and serum-starved overnight. Following that, HeLa cells were treated with 1 µM FITC-labeled S-20 or S-20-1, respectively, for 2 h and then washed with PBS buffer three times. Next, the cells were fixed with MeOH for 5 min at room temperature, followed by washing with PBS three more times. Cells were then incubated with 1 µg/mL DAPI/PBS for 15 min in the absence of light, followed by thoroughly washing with PBS again. Finally, cells were observed by the inverted Nikon fluorescence microscope.

2.5.4 Fluorescence polarization assay

50 nM FITC-labeled γ-AApeptides were incubated with protein (0-2 µM) in PBS. Dissociation constants (Kd) were determined by plotting fluorescence anisotropy values as a function of protein concentration, and the plots were fitted to the following equation.

\[ y = \left[ FP_{\text{min}} + (FP_{\text{max}} - FP_{\text{min}}) \right] \]

\[
\frac{(K_d + L_{st} + x) - \sqrt{(K_d + L_{st} + x)^2 - 4L_{st}x}}{2L_{st}}
\]
\( L_{st} \) and \( x \) refer to the concentration of the peptide and protein, respectively. The experiments were conducted in triplicate and repeated three times.

### 2.5.5 Molecular docking studies

Molecular docking studies were carried out as previously described.\(^3\) The molecular docking of \textbf{S-20} toward RBD and HR1 was carried out using the Schrödinger Glide program. The conformational search of \textbf{S-20} was performed using mixed torsional/low-mode sampling as implemented in Schrödinger (2015) with AMBER force field. The RBD (PDB: 6M0J) and HR1 (PDB: 7C53) of SARS-CoV-2 were chosen for docking. After removal of water and redundant small molecules using PyMol, the proteins were prepared using Schrödinger Protein Preparation Wizard with default settings. Grids were generated using the centroid of the interaction surface as the centers for docking. Docking was performed using the Glide module in Schrödinger (2015) with default parameters.

### 2.5.6 PAMPA-BBB assay

Following a previous report,\(^3\) the PAMPA-BBB assay procedure was developed by pION. All liquid handling steps were performed on the TECAN Freedom EVO150 robot and analyzed by Pion PAMPA Evolution software. BBB PAMPA included brain the sink buffer (BSB), lipid solution (BBB-1) and Stirwell\textsuperscript{TM} PAMPA Sandwich plate preloaded with magnetic stirring disks. 4 \( \mu \)L of lipid solution were transferred into the acceptor well to which 200 \( \mu \)L of BSB (pH 7.4) were added. Then, 180 \( \mu \)L of diluted test compounds (50-250 \( \mu \)M in system buffer at pH 7.4 from a 10 mM DMSO solution) were added to the donor wells. The PAMPA sandwich plate was assembled, placed on the Gut-Box\textsuperscript{TM} and stirred with 60 \( \mu \)m Aqueous Boundary Layer (ABL)
settings for 1 h incubation. Distribution of compounds in the donor and acceptor buffer (150 µL aliquot) was determined by UV spectra measurement from 250 to 498 nm using the TECAC Infinite M-1000 Pro microplate reader. Permeability ($P_{app}$, 10$^{-6}$ cm/s) of each compound was calculated by Pion PAMPA evolution software. The assay was performed in triplicate.

### 2.5.7 PAMPA-GIT assay

PAMPA-GIT assay was also realized by using a method developed by pION.$^{30}$ We also used the TECAN Freedom EVO150 robot to perform all liquid handling steps and analyzed the data by pION’s PAMPA Evolution software. The pION’s GIT PAMPA includes the acceptor sink buffer (ASB), GIT-0 Lipid solution and the Stirwell$^{TM}$ PAMPA sandwich plate preloaded with magnetic disks. Four µL of lipid were transferred in the acceptor well, followed by addition of 200 µL of ASB (pH 7.4). Then, 180 µL of diluted test compound (50-250 µM in system buffer at pH 5.0, 6.2 and 7.4 from a 10 mM DMSO solution) were added to the donor wells. The PAMPA sandwich plate was assembled and placed on the Gur-Box$^{TM}$ and stirred with 40 µm Aqueous Boundary Layer (ABL) settings for 30 min. Distribution of the compounds in the donor and acceptor buffers (150 µL aliquot) was determined by UV spectra measurement from 250 to 498 nm using the TECAN Infinite M-1000 Pro microplate reader. Then the Permeability ($P_e$, cm.s$^{-1}$) of each compound was calculated by Pion PAMPA evolution software. The assay was performed in triplicate.

### 2.5.8 Assessment of enzymatic stability of S-20-1

Cyclic $\gamma$-AA peptides S-20-1 (0.1mg/mL) were incubated with 0.1 mg/mL protease in 100 mM ammonium bicarbonate buffer (pH 7.8) at 37 °C for 24 h. After that, water and ammonium
bicarbonate in the reaction mixtures were removed using the speed vacuum. The residues were dissolved in 100 µL H₂O/ACN and analyzed on a Waters analytical HPLC system.

2.5.9 *Inhibition of pseudovirus infection*

Assays for measuring the inhibitory activity of the compounds against pseudotyped coronavirus infection were conducted as previously described⁴³,⁴⁴. Plasmids encoding spike protein of coronavirus, including SARS-CoV-2, SARS-CoV-2 variants (Alpha, Beta, gamma, lambda, Delta, Omicron), SARS-CoV, MERS-CoV, HCoV-OC43, HCoV-229E, SARSr-CoV WIV1, luciferase reporter vector (pNL4-3. Luc.R-E-), and plasmids encoding EGFP were maintained in our laboratory. For the package of pseudoviruses, pcDNA3.1- SARS-CoV-2-S and pNL4-3. Luc.R-E- were co-transfected into 293T cells using Vigofect transfection reagent, and then the supernatants were changed with fresh medium containing 10% FBS. After 48h, the supernatants containing pseudoviruses were collected, filtered with a 0.45 µm filter, and stocked. To determine the inhibitory activity of a given compound, target cells (Huh-7 cells) were seeded at 8000 per well in a 96-well plate and cultured at 37 ℃ for 12 hours. The compound was diluted with DMEM without FBS, and then the same volume of pseudoviruses was added. Afterwards, the mixture was transferred into Huh-7 cells and incubated for 30 min. After 12 hours, the mixture was replaced with fresh medium. Forty-eight hours later, the cells were lysed with cell lysis buffer, and luciferase activity was detected with the Luciferase Assay System (Promega, Madison, WI, USA).
2.5.10 Inhibition of authentic coronavirus infection

The inhibitory activity of S-20-1 against authentic viruses was tested according to previous study. In brief, S-20-1 was serially diluted with DMEM without FBS. Then 100 TCID50 viruses were mixed with diluted S-20-1. After incubation for 30 min, the mixtures were transferred to target cells (RD for HCoV-OC43, Huh-7 for HCoV-229E, and Caco-2 for SARS-CoV-2 and SARS-CoV-2 Delta). The medium was changed 12 hours later, and cell viability was detected with CCK8 kit (HCoV-OC43 and HCoV-229E). For SARS-CoV-2 and SARS-CoV-2 Delta, the supernatants were collected after 48 hours. The viral RNA load was tested as previously reported. Briefly, the viral RNA was extracted with RNA extraction kit (Transgene, China). Then the N gene of SARS-CoV-2 was tested by real-time RT-PCR. The sequence of primer and probe follow:

Forward: GGGGAACCTTCTCCTGCTAGAAT;

Reverse: CAGACATTTTTGCTCTCAAGCTG

Probe: 5'-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3'

2.5.11 Inhibition of S protein-mediated cell-cell fusion

The cell-cell fusion assay was established and performed as in previous study. In brief, PAAV-IRES-EGFP S was transfected to 293T cells to obtain effector cells expressing S protein of coronaviruses, including SARS-CoV-2, SARS-CoV, MERS-CoV, HCoV-229E and HCoV-NL63, and GFP. Then serially diluted S-20-1 was mixed with effector cells, and the mixture was transferred to Huh-7 cells (target cells). For SARS-CoV and NL63 S-mediated cell-cell fusion assay, trypsin (80 mg/ml) was added to the mixture. After incubation for 2-4 hours, fused cells were counted, and the fusion rate was calculated to determine inhibitory activity.
2.5.12 Time-of-addition assay and time-of-removal assay

As in previous study,\textsuperscript{38,47} for time-of-addition assay, Huh-7 cells (for pseudotyped SARS-CoV-2) and RD cells (for HCoV-OC43) were seeded into a 96-well plate at 10,000 per well, respectively. \textbf{S-20-1} was added at the final concentration of 50 µM 0.5h before or 0, 0.5, 1, 2, 4, 6, and 8h after addition of SARS-CoV-2 pseudoviruses or HCoV-OC43 (100 TCID50). The inhibitory activity of \textbf{S-20-1} was determined as described above.

For time-of-removal assay, \textbf{S-20-1} was added to Huh-7 cells to incubate at 37 ℃ for 1 hour. After \textbf{S-20-1} was removed, SARS-CoV-2 pseudovirus was added to infect cells. \textbf{S-20-1} was not removed from the group set as control. The medium was changed 12 hours later, and luciferase activity was tested as described above.

2.5.13 Assays for detecting viral entry, attachment, post-attachment, and post-entry

Viral entry assay was performed as previously described,\textsuperscript{38,47} Briefly, \textbf{S-20-1} and virus were added to target cells at 37 ℃ for 1 hour, and then cells were washed with cold PBS three times. To perform the viral attachment assay, the mixture of \textbf{S-20-1} and virus was added to target cells to incubate for 1 hour at 4 ℃ before washing with cold PBS. For the post-attachment assay, virus was incubated with target cells at 4 ℃ for 1 hour. Then the cells were thoroughly washed with cold PBS to remove the unattached virus. After this, \textbf{S-20-1} was added and incubated at 37 ℃ for an additional 1 hour. The post-entry assay was performed like the post-attachment assay, except that virus was incubated with cells at 37 ℃. The inhibition effects of \textbf{S-20-1} were detected as above.
2.5.14 Cytotoxicity assay

Cytotoxicity of compound to cells (Huh-7 cells and Caco-2 cells) was tested as previously described. Briefly, serially diluted compounds were added to target cells. After culture at 37 °C for 12 hours, the medium was changed with fresh medium. Forty-eight hours later, the supernatant was removed, and cell viability was analyzed with Cell Counting Kit (CCK-8). In a 96-well plate, 100 µL of diluted CCK8 reagent were added to each well, and the absorbance was measured at 450 nm.

2.5.15 Mouse pharmacokinetic studies

In two separate experiments, S-20-1 was administered either p.o. or i.p. to mice at the dose of 50 mg/kg, volume 150 µL. Following administration, 100 µL blood samples were collected at 10 min, 20 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and 48 h (n=3 per time point, and each mouse was used for three time points; thus 9 mice were used for either p.o. or i.p., making a total of 18 mice). After drug administration, 100 µL of blood were collected into 1.5-mL Eppendorf tubes containing 30 µL disodium EDTA (0.5 M, pH 8.0) and kept on ice until plasma collection (< 30 min), followed by centrifugation at 4,000 rpm/min for 10 min at 4 °C. sera were collected and stored at -80 °C for future analysis. Serum samples of 50 µL were added to 135 µL acetonitrile and 15 µL glacial acetic acid. Samples were allowed to rest on ice for 15 min and then centrifuged at 10,000 rpm and 4 °C for 15 min. Clarified supernatants were transferred to vials and analyzed by LC/MS/MS. PK parameters were obtained using PKSolver.
2.5.16 Evaluation of the in vivo protective activity of S-20-1

The protective effect of S-20-1 against coronavirus in vivo was performed according to previous study.22 Animal studies were approved by the Institutional Laboratory Animal Care and Use Committee at Fudan University (Approval number: 20200821-002). For HCoV-OC43, infected newborn mice were established as previously described. Pregnant Balb/c mice (18 days) were separated into three groups after delivery of their offspring. Each group contained seven newborn mice. For mice in the prophylactic and therapeutic groups, S-20-1 was administered through the intranasal route at 80mg/kg before or after challenge with HCoV-OC43. At the 4th day post-infection, the newborn mice were dissected. The relative viral RNA expression level in brain was tested through RT-PCR and calculated as $2^{(-\Delta\Delta C_t)}$. The HCoV-OC43 RNA level was adjusted with mouse housekeeping gene GAPDH. The primer of HCoV-OC43 and GAPDHA follows:

OC43-S-Forward: GACACCGGTCCCTCCTCCTAT;
OC43-S-Reverse: ACACTTCCCTTCAGTGCCAT;
GDPAH-Forward: TGCTGTCCCTGTATGCCTCTG;
GDPAH-Reverse: TTGATGTCACGCACGATTTCC

For SARS-CoV-2 Delta, we used C57BL/6-Tgtn (CAG-human ACE2-IRES-LuciferaseWPRE-polyA) transgenic mice infected with SARS-CoV-2.37 Eight- week-old female hACE2 transgenic mice were challenged with SARS-CoV-2 Delta variant at 10000 pfu via the intranasal route. For prevention and therapy groups, S-20-1 was administered at the dose of 60mg/kg through the intranasal route 30 min before or after viral challenge. Then the mice were euthanized at 4 days post-infection, and brains, lungs and intestines were dissected. Viral RNA
was extracted with TRIzol reagent according to the manual. Real-time RT-PCR was conducted to evaluate viral RNA load in tissues as described previously.

2.5.17 Evaluation of in vitro proteolytic enzyme stability and in vivo safety of S-20-1

For stability, the resistance of S-20-1 to proteinase K and trypsin was performed as previously described.44 S-20-1 was incubated with proteinase K (1 microunit/ml) for different time and then centrifuged at 500 g for 5 min to remove the proteinase K. To determine the stability of S-20-1 against trypsin, S-20-1 was incubated with trypsin (25 mg/ml) for different time, followed by addition of FBS to final proportion of 20% and heated at 56 ℃ for 30 min to inactivate trypsin. The inhibitory activity of treated S-20-1 was tested on Huh-7 cells.

Eight-week-old mice (two groups, n=6) were used to evaluate the safety of S-20-1 in vivo. According to previous study,48 100 mg/kg S-20-1 were intranasally administered to mice daily for three consecutive days. Then bodyweight was monitored for 12 days, followed by observing the behavior of mice. At 12 days, mice were euthanized to harvest the brains, lungs, livers, and kidneys for hematoxylin and eosin staining.

2.5.18 Statistical analysis

Student’s \( t \) test and Analysis of Variance (ANOVA) were used to compare the difference by GraphPad Prism in this manuscript. * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), and **** \( P < 0.0001 \).
2.5.19 Characterization of macrocyclic $\gamma$-AApeptides

a. HPLC analytic trace of S-20.

b. HPLC analytic trace of S-20-I.

c. HPLC analytic trace of FITC-S-20.

d. HPLC analytic trace of FITC-S-20-I.

Figure 16 HPLC analytic trace
### Table 1 Inhibitory activity, cytotoxicity, and selective index (SI) of the 7 lead compounds

<table>
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<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
<th>CC₅₀ (µM)</th>
<th>SI</th>
<th>Compound</th>
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<th>CC₅₀ (µM)</th>
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### Table 2 Inhibitory activity, cytotoxicity, and selective index (SI) of the 4 selected compounds

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<th>CC₅₀ (µM)</th>
<th>SI</th>
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<td>S-25-1</td>
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<td>&gt; 481.93</td>
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### Table 3 HRMS of all compounds including FITC labeled compounds

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<th>HRMS (ESI) ([M+2H]²⁺) Found</th>
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<td>1,101.0040</td>
<td>2,200.6440</td>
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</table>
2.6 References


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Chapter 3  An HR2-mimicking Sulfonyl-γ-AApeptide is A Potent Pan-coronavirus Fusion Inhibitor with Strong Blood-brain Barrier Permeability, Long Half-life and Promising Oral Bioavailability

Note to Reader

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3.1 Introduction

COVID-19, caused by SARS-CoV-2, is associated with more than 663 million confirmed cases and 6.7 million deaths as of 7 January 2023.\(^1\) Although several vaccines\(^2\) and small-molecule drugs\(^3\) have now been authorized or approved for human use, the consistent emergence of new viral variants has quickly jeopardized their efficacy.\(^4\) Therefore, it is imperative to continue developing alternative and broad-spectrum prophylactics and therapeutics to combat this pandemic.

SARS-CoV-2 is a member of the group of highly mutable β-coronaviruses, enabling them to adapt to new hosts and ecological niches.\(^5\) Its viral particles are composed of four structural proteins: spike (S), envelope (E), membrane (M), and nucleoprotein (N) proteins.\(^6\) These proteins play significant roles in the viral life cycle and are common to all human coronaviruses (HCoVs), making them prospective targets for the development of broad-spectrum antiviral agents.\(^7\) Among them, S protein facilitates viral entry into target cells by recruiting the cellular serine protease
TMPRSS2 for S protein priming and the angiotensin-converting enzyme 2 (ACE2) as the entry receptor (Figure 17a).\textsuperscript{8,9} S protein has two functional subunit domains (Figure 17a and 17b), S1 and S2.\textsuperscript{10} The S1 subunit binds with the ACE2 receptor through its receptor-binding domain (RBD), followed by conformational changes in the S2 subunit that allow the fusion peptide domain (FP) to insert into the cell membrane of host target cells. The heptad repeat 1 (HR1) region in the S2 subunit assembles into a homotrimeric structure, exposing three highly conserved hydrophobic grooves that interact with heptad repeat 2 (HR2) (Figure 17c and 17d) to form a 6-HB structure that brings the viral and cellular membranes to close together to start the viral fusion process (Figure 17b).\textsuperscript{7,11} The RBD and HR regions are excellent targets for the development of specific therapeutics aimed at the fusion process.\textsuperscript{12} Although RBD-based antibodies have been validated to effectively prevent virus attachment to ACE2,\textsuperscript{13} it is extremely challenging to design a broad-spectrum antiviral drug that targets the RBD owing to its highly mutable nature.\textsuperscript{14,15} In contrast, it is generally agreed that HR1 could serve as a good target for the development of pan-CoV fusion inhibitors against highly pathogenic HCoVs since it is highly conserved among diverse HCoVs.\textsuperscript{7,16}

A few peptide-based fusion inhibitors targeting HR1 have now been developed, and they have shown considerable effectiveness in preventing SARS-CoV-2 infection \textit{in vitro} and \textit{vivo}.\textsuperscript{7,16-21} Jiang and colleagues identified two pan-CoV fusion inhibitors, EK1\textsuperscript{17} and EK1C4,\textsuperscript{16} which could significantly inhibit the fusion of diverse HCoVs, including SARS-CoV-2, SARS-CoV, MERS-CoV, HCoV-NL63, and HCoV-OC43. Zhu et al.\textsuperscript{17} created a lipopeptide fusion inhibitor, IPB02, which was highly effective in preventing SARS-CoV-2 S protein-mediated cell-cell fusion and pseudovirus infection. Daily intranasal treatment of [SARSHRC-PEG4]\textsubscript{2}-chol, a dimeric lipopeptide fusion inhibitor developed by de Vries et al.,\textsuperscript{18} to SARS-CoV-2 ferrets could entirely stop SARS-CoV-2 direct-contact transmission. However, these bioactive peptides have intrinsically low
biostability and bioavailability owing to their canonical peptide backbone, which results in a short half-life and makes them challenging to use as oral medications.\textsuperscript{22}

\textbf{Figure 17 Fusion process of SARS-CoV-2 infection and proposed inhibitory mechanism of sulfonyl-γ-AApeptides}

(a) Schematic representation of SARS-CoV-2 spike protein. SP, signal peptide; NTD, N-terminal domain; RBD, receptor-binding domain; RBM, receptor-binding motif; FP, fusion peptide; HR1, heptad repeat 1; HR2, heptad repeat 2; TM, transmembrane domain; CP, cytoplasm domain. (b) Fusion process mediated by S protein of SARS-CoV-2 and proposed mechanism of sulfonyl-γ-AApeptides to inhibit the infection of SARS-CoV-2. (c) Side view of the crystal structure of 6-HB formed by the association between HR2 and HR1 (PDB code 6LXT). (d) Binding interaction of key residues on HR2 (green) with the pockets on HR1 trimer.
Non-natural sequence-specific peptidomimetics have become a promising alternative strategy to modulate protein-protein interactions (PPIs)23-27 to alleviate issues associated with the intrinsic drawbacks of peptides. In addition to retaining the advantages of natural peptides, foldameric peptidomimetics also exhibit unique structures and functions and are highly resistant to enzymatic hydrolysis.23,28 We have developed a new class of peptidomimetics, γ-AApeptides28-30 (oligomers of N-acylated-N-aminoethyl amino acids), based on the γ-chiral PNA backbone. They show extraordinary resistance to proteolytic degradation and amenability to chemical diversification, making them suitable candidates for a variety of biological applications.12,31-34 As a subclass of γ-AApeptides, sulfonyl-γ-AApeptides (Figure 18a) not only possessed the merits noted above, but they could also adopt well-defined helical structures33-36 (Figure 18b and 18c). Notably, the sulfonyl-γ-AApeptide helix displays a more robust and stable helical conformation than the α-helix of the same length, presumably from the intramolecular hydrogen bonding and the curved nature of sulfonamide moieties in the molecular framework. It is well known that homogeneous L-sulfonyl-γ-AApeptides adopt left-handed 414-helix helical conformations with 4 side chains per turn and helical pitch of 5.1 Å35 (Figure 18b and 18c). Since these are characteristics analogous to α-helix, albeit with different handedness, they have been designed to mimic the helical domain of proteins and have been shown to effectively modulate a number of PPIs, such as BCL9,37 p53,38 GLP-139 and VEGF.40,41 It should be noted that the left-handedness in the sequences could be switched to right-handedness by changing L-sulfonyl-γ-AA building blocks to D-sulfonyl-γ-AA building blocks (Figure 18d), leading to right-handed helices expected to further facilitate the design of mimetics of α-helix owing to their closer similarity (Figure 18e and 18f).36,42 We envisioned that the molecular scaffold of D-sulfonyl-γ-AApeptide could be adopted, through rational design, to inhibit the viral fusion process of SARS-CoV-2.
To the best of our knowledge, no fusion inhibitors based on entirely unnatural foldameric scaffolds have yet been reported to block the entry of SARS-CoV-2. Herein we report the design of right-handed helical D-sulfonyl-γ-AApeptides that mimic the hot spots of the HR2 peptide and disrupt the interaction between the HR1 and HR2 domains of the S2 subunit in SARS-CoV-2. By conjugating a cholesterol molecule to the lead sulfonyl-γ-AApeptides, we identified a lipo-sulfonyl-γ-AApeptide (XY4-C7) which exhibited potent inhibitory activity against SARS-CoV-2 in the pseudovirus (PsV) and authentic virus infection assays, presenting a high selectivity index (SI). Additionally, XY4-C7 demonstrated broad-spectrum antiviral activity against a range of coronaviruses, including SARS-CoV-2 and its Delta variant and other HCoVs like SARS-CoV, MERS-CoV, HCoV-NL63, and HCoV-OC43, as well as bat SARS-related coronavirus (SARSr-CoV) WIV1, which is consistent with the fact that both HR2 and HR1 domains are highly conserved across divergent coronaviruses. Moreover, administration of XY4-C7 via the nasal route revealed highly prophylactic and excellent therapeutic effects in vivo studies. Additionally, owing to its unnatural backbone, XY4-C7 showed remarkable resistance to proteolytic degradation and demonstrated a very long half-life and promising oral bioavailability in PK studies, suggesting that sulfonyl-γ-AApeptide has the potential to be developed into therapeutic and prophylactic drugs for the treatment and prevention of infection by SARS-CoV-2 and other HCoVs.

3.2 Results

3.2.1 Structure insight of six-helical bundle (6-HB) formed between HR1 and HR2

The 6-HB formed by HR1 and HR2 domains is crucial to the membrane fusion mediated by SARS-CoV-2 S protein, and its crystal structure has recently been determined.16 As shown in Figure 17c and 17d, three HR1 molecules form a parallel trimeric coiled-coil center, which is
surrounded by three antiparallel HR2 helices. Hydrophobic force drives the interaction between HR1 and HR2 domains, and this interaction is mainly located in the helical fusion core region. Each pair of two neighboring HR1 helices creates a substantial hydrophobic groove that serves as the binding site for hydrophobic residues from the HR2 domain, including I1179, I1183, L1186, V1189, L1193 and L1197, which are on the same face of HR2 α-helix (Figure 17d). Additionally, side chain-to-side chain hydrophilic interactions also stabilized the bundle structure.

![Figure 18 Chemical and crystal structure of sulfonyl-γ-AApeptides](image)

3.2.2 Rational design of sulfonyl-γ-AApeptides to mimic HR2 peptide in the fusion core

Based on the above analysis, we introduced 6 chiral hydrophobic residues at the 1a, 3a, 5a, 7a, 9a, and 11a positions on the same face of D-sulfonyl-γ-AA peptide helices to mimic the binding interaction of I1179, I1183, L1186, V1189, L1193 and L1197 of HR2, respectively, to reproduce binding affinity with HR1 (Figure 18g and 18h). Negatively and positively charged side chains were introduced to the sequences to form the salt bridges in the other two faces to enhance the
helical stability and solubility of the sequences (Table 4). We first designed and synthesized four sequences (XY1-XY4, Table 4) bearing different sizes of hydrophobic groups at position 1b, 8b and 10b, as these residues reside in the hydrophobic groove of HR1. We first performed fluorescence polarization assays to evaluate the binding affinity of these sequences toward the HR1 peptide (911-987). As expected, all four sequences showed an excellent binding affinity with the HR1 peptide with Kd values from 0.13 μM to 0.42 μM (Table 4). This is consistent with the modeling in which the crucial residues of HR2 (Figure 19a) and the hydrophobic side chains of XY4 (Figure 19b) overlap very well (Figure 19c). The overlay of XY4 with the HR2 peptide on the surface of HR1 (Figure 19d) also suggested that XY4 could recognize the hydrophobic cleft of HR1 effectively. Indeed, all D-sulfonyl-γ-AApeptides assumed typical right-handed helical structures in solution. As shown in Figure 19e, CD experiments were carried out and revealed strong negative cotton effects between 205 and 215 nm, which is a mirror image of the CD signature of left-handed L-sulfonyl-γ-AApeptides, implying that these D-sulfonyl-γ-AApeptides adopted right-handed helical conformations, which is similar to α-helical peptides.

Next, we tested the inhibitory activity of these four sequences at the concentration of 20 μM against SARS-CoV-2 infection in vitro, using our well-established SARS-CoV-2 PsV infection assay with Caco-2 cells. While not highly potent, all of them exhibited a certain level of inhibitory activity, particularly compound XY4, which inhibited PsV infection by roughly 40% at this concentration (Figure 19f). We also synthesized XY5, XY6, XY7, and XY8 in which certain hydrophobic residues at positions 1a, 3a, 5a, 7a, 9a, and 11a were changed to hydrophilic groups (Table 4). As expected, their binding affinity with the HR1 peptide was very low, confirming that potent binding affinity results from successful mimicry of the critical hydrophobic residues in the
HR2 peptide in the fusion core by sulfonyl-γ-AApeptides. As XY4 displayed the most effective inhibitory activity, it was selected as our lead compound for further modification.

Figure 19 Rational design of sulfonyl-γ-AAPeptide-based entry inhibitors and evaluation of their inhibitory activity in vitro

(a) Structure of HR2 peptide in fusion core (white). (b) Sulfonyl-γ-AAPeptide mimic XY4 (green). (c) Overlay of key binding residues between (a) and (b). (d) Superimposition of XY4 (green) with critical residues of HR2 peptide in fusion core (white) on the binding surface of HR1. (e) CD spectra of sulfonyl-γ-AApeptides XY1-XY4 and XY4-C7 measured at 100 μM, room temperature, in PBS buffer. (f) Inhibitory activity of 4 lead compounds (XY1, XY2, XY3, and XY4) at the concentration of 20 μM from SARS-CoV-2 pseudovirus infection assay. (g)-(j) Binding affinity of binding between HR1 peptide and XY1 (g), XY2 (h), XY3 (i), and XY4 (j) determined by fluorescence polarization.
3.2.3 **XY4-C7, sulfonyle-γ-A4peptides-PEGn-Chol, demonstrated excellent fusion inhibitory activity and moderate cytotoxicity**

**XY4** could inhibit SARS-CoV-2 infection; however, its activity is considerably weaker than that of the recently reported pan-CoV fusion inhibitors, EK1. Lipidation is a demonstrated strategy to enhance the antiviral activities of fusion inhibitors such as EK1C4 by increasing their local concentration at the host cell’s membrane surface. Therefore, cholesterol (Chol) was covalently attached to the C-terminus of **XY4** with the assistance of different spacers, and the corresponding **Chol-PEGn-XY4s** were constructed (Figure 20a). These **Chol-PEGn-XY4s** were evaluated by SARS-CoV-2 PsV infection assay as indicated by half maximal inhibitory concentration (IC50). First, we added two flexible linkers, (Fmoc-6-aminohexanoic acid) and Chol-polyethylene glycol (PEG), with varied lengths of 4, 8, and 12 units. The IC50 values of these three compounds were determined to be 29.820 µM, 2.857 µM, and 0.925 µM, respectively (Figure 20b), indicating that lipidation modification was successful and that more units of PEG added would increase the inhibitory activity. We changed flexible linkers to γSγS (Figure 20a) to obtain the other four sequences **XY4-C4, XY4-C5, XY4-C6, and XY4-C7**, which were inspired by the rigid linker (GSGSG) utilized by EK1C4. The anti-PsV activity of these four compounds was examined in Caco-2 cells, and the IC50 values for each were found to be 35.77 µM, 4.05 µM, 2.63 µM, and 0.79 µM, respectively (Figure 20b), among which **XY4-C7** appeared to be much more potent than **XY4**, exhibiting even a better activity than the previously reported EK1 peptide (IC50: 2.38 µM). As a result, we chose **XY4-C7** as the lead compound to do further assessments. It is worth noting that **XY4-C7** effectively prevented authentic SARS-CoV-2 infection at the cellular level in a dose-dependent manner with an IC50 of 0.24 µM in Caco-2 cells (Figure 20c), consistent with the results from the PsV infection assay. The same cell line was used to evaluate its
cytotoxicity, and the half-maximal cytotoxic concentration (CC_{50}) was 14.89 µM (Figure 20e). The selectivity index (SI=CC_{50}/IC_{50}) was 62.04, suggesting that XY4-C7 specifically inhibits SARS-CoV-2 entry into the host cells. The subsequent experiment also demonstrated that XY4-C7 targeted the S protein and inhibited the SARS-CoV-2 S protein-mediated cell-cell fusion in a dose-dependent manner (Figure 20d). The binding affinity of XY4-C7 toward the HR1 peptide was determined by both fluorescence polarization assay and isothermal titration calorimetry (ITC) assay with IC_{50} values of 0.1 µM and 0.8 µM, respectively, suggesting that adding Chol to XY4 did not significantly change the binding activity to the HR1 peptide (Figure 20f-h). After that, we employed circular dichroism (CD) to probe the mechanism of the inhibitory activity of XY4-C7. Both HR1 peptide and HR2 peptide exhibited the typical α-helicity in the solution; the mixture of HR1 and HR2 peptide show a more pronounced α-helical character (Figure 20i), which may indicate the formation of the HR1/HR2 complex. However, in the presence of XY4-C7, the intensity of the CD signature of the HR1 peptide decreased dramatically, implying a significant conformational change due to the interaction between the HR1 peptide and XY4-C7. In addition, the characteristic of α-helicity of 6-HB significantly decreased when mixing XY4-C7 with HR1 peptide/HR2 peptide together (Figure 20j), suggesting that XY4-C7 could disrupt the formation of HR1/HR2 complex by potently binding with the HR1 (Figure 20f, 20g, 20h, and 20j). Taken together, XY4-C7 is a potent and selective inhibitor of SARS-CoV-2 infection with a high binding affinity toward the HR1 peptide to disrupt the formation of 6-HB between HR1 and HR2 fusion core.
Figure 20 Rational design of sulfonyl-γ-AApeptides-PEGn-Chol inhibitors and evaluation of their antiviral activity *in vitro*

(a) Design diagram of sulfonyl-γ-AApeptides-PEGn-Chol, including XY4C1-XY4-C7. (b) Inhibitory activity of sulfonyl-γ-AApeptides-PEGn-Chol on SARS-CoV-2 PsV infection. (c) Inhibitory activity of XY4-C7 on authentic SARS-CoV-2 infection in Caco-2 cells. (d) Inhibitory activity of XY4-C7 on SARS-CoV-2 S-mediated cell-cell fusion. (e) Cytotoxicity of XY4-C7 to Caco-2 cells. (f) Affinity of binding between XY4-C7 and the HR1 peptide determined by fluorescence polarization. (g) and (h) Affinity of binding between XY4-C7 and the HR1 peptide determined by isothermal titration calorimetry (ITC). (i) CD spectra of SARS-CoV-2 HR1 peptide alone (orange), SARS-CoV-2 HR2 peptide (navy) and SARS-CoV-2 HR1/HR2 peptide complex (dark yellow). (j) CD spectra of XY4-C7/HR1 peptide mixture (black), XY4-C7/HR1 peptide/HR2 peptide mixture (red) and SARS-CoV-2 HR1/HR2 peptide complex (blue).
3.2.4 *XY4-C7 efficiently inhibited infection by authentic SARS-CoV-2 Delta variant, and 4 pseudotyped HCoVs and 1 pseudotyped bat SARSr-CoV*  

To determine the breadth of *XY4-C7*, we tested its inhibitory activity against the SARS-CoV-2 Delta variant, 4 other HCoVs, and one bat SARSr-CoV. We found that *XY4-C7* is effective against authentic SARS-CoV-2 Delta variant infection in Vero-E6 cells with an IC₅₀ value of 4.73 µM (Figure 21a). *XY4-C7* could also potently inhibit infection of pseudotyped SARS-CoV, MERS-CoV, and HCoV-NL63 as well as bat SARSr-CoV WIV1 in different cell lines with IC₅₀s ranging from 0.81 to 9.42 µM, confirming that *XY4-C7* is a pan-HCoV fusion inhibitor (Figure 21b-21e). Overall, *XY4-C7* is a promising broad-spectrum antiviral agent that is effective against SARS-CoV-2 and Delta variant as well as other HCoVs and bat SARSr-CoV that may cause future coronavirus diseases.

3.2.5 *Intranasally applied XY4-C7 potently protected newborn mice against HCoV-OC43 infection*  

We next employed a mouse model of HCoV-OC43 infection to investigate the protective efficacy of *XY4-C7* in clinical applications. *XY4-C7* was administered to OC43-infected newborn mice in prevention (n = 5) or treatment (n = 5) groups via the intranasal route at a low-single dose of 1 mg/kg 0.5 h before or after challenge with HCoV-OC43 at 100 TCID₅₀, respectively. Mice were sacrificed after 4 days, and brains were excised to determine viral load. As shown in Figure 21g, both prevention and treatment groups revealed significantly lower HCoV-OC43 RNA levels than the non-treatment group. This result suggests that *XY4-C7* can effectively protect newborn mice from infection of HCoV-OC43, and based on this evidence, it is plausible to anticipate that
XY4-C7 could effectively inhibit SARS-CoV-2 infection, as well as infection by other HCoVs in vivo.

**Table 4 Structure of selected sulfonyl-γ-AApeptides helical mimics (XY1-XY8)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Kd (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XY1</td>
<td><img src="image1.png" alt="Image" /></td>
<td>0.28</td>
</tr>
<tr>
<td>XY2</td>
<td><img src="image2.png" alt="Image" /></td>
<td>0.32</td>
</tr>
<tr>
<td>XY3</td>
<td><img src="image3.png" alt="Image" /></td>
<td>0.42</td>
</tr>
<tr>
<td>XY4</td>
<td><img src="image4.png" alt="Image" /></td>
<td>0.13</td>
</tr>
<tr>
<td>XY5</td>
<td><img src="image5.png" alt="Image" /></td>
<td>NA</td>
</tr>
<tr>
<td>XY6</td>
<td><img src="image6.png" alt="Image" /></td>
<td>NA</td>
</tr>
<tr>
<td>XY7</td>
<td><img src="image7.png" alt="Image" /></td>
<td>NA</td>
</tr>
<tr>
<td>XY8</td>
<td><img src="image8.png" alt="Image" /></td>
<td>NA</td>
</tr>
</tbody>
</table>

*Critical binding residues were shown in red. The binding affinity (Kd) of sequences to the HR1 peptide was determined by FP. The amino acid sequence of the HR2 peptide in the fusion core is SVVNIQKEIDRLNEVKKNLNESSLDLQ.*
3.2.6 **XY4-C7 was highly stable in the presence of pronase or human sera**

The inherent susceptibility to degradation toward proteolytic enzymes is a major bottleneck for canonical peptides for the development of antiviral agents. To this end, we evaluated the stability of XY4-C7 and the HR2 peptide in the presence of pronase, a mixture of hydrolytic enzymes theoretically degrading peptides into single amino acids or human sera. The samples were incubated with pronase or human sera for 24 h and analyzed with LC/MS/MS (Figure 22a). XY4-C7 was highly stable and did not show noticeable degradation in 24 h; however, the HR2 peptide was completely degraded in pronase and around 90% degraded in human sera.

3.2.7 **XY4-C7 demonstrated favorable passive permeability to the blood-brain barrier (BBB) and gastrointestinal tract (GIT) membranes**

The parallel artificial membrane permeability assay (PAMPA) is a type of high-throughput permeability assay that has been widely used in the pharmaceutical industry to assess drug candidates. To this end, we assessed XY4-C7 with PAMPA for BBB permeability (PAMPA-BBB) and the PAMPA for gastrointestinal tract parallel artificial membrane permeability (PAMPA-GIT). Expected $P_{app}$ values for favorable, medium, and low permeability are $> 20 \times 10^{-6}$ cm/s, 1–20 $\times 10^{-6}$ cm/s, and $< 1 \times 10^{-6}$ cm/s, respectively, in both assays. Since human intraluminal pH varies in the stomach, duodenum, ileum, cecum, and rectum, we needed to evaluate the permeability capacity of XY4-C7 under various pH conditions for the PAMPA-GIT. We used the fully orally bioavailable drug Carbamazepine (50 µM) as the positive control, which had higher $P_{app}$ values with around $150 \times 10^{-6}$ cm/s at three different pH values, and the poorly orally bioavailable drug Antipyrine (200 µM) as the negative control, which had lower $P_{app}$ values $< 6 \times 10^{-6}$ cm/s at the same pH values (Figure 22b). As shown in Figure 22b, XY4-C7 (50 µM)
displayed favorable permeability with much higher $P_{app}$ values of $372 \times 10^{-6}$ cm/s, $344 \times 10^{-6}$ cm/s, and $306 \times 10^{-6}$ cm/s at pH 5.0, 6.2 and 7.4, respectively. As a result, XY4-C7 is expected to have very promising oral bioavailability \textit{in vivo}.

\textbf{Figure 21 Broad-spectrum antiviral activity of XY4-C7 \textit{in vivo} and \textit{vivo}}

(a) Inhibitory activity of XY4-C7 against infection of authentic SARS-CoV-2 Delta variant in Vero-E6 cells. (b) Inhibitory activity of XY4-C7 against pseudotyped HCoV-NL63 in ACE2/293T cells. (c) Inhibitory activity of XY4-C7 against pseudotyped SARS-CoV in Huh-7 cells. (d) Inhibitory activity of XY4-C7 against pseudotyped MERS-CoV in Huh-7 cells. (e) Inhibitory activity of XY4-C7 against pseudotyped bat SARSr-CoV W1V1 in ACE2/A549 cells. (f) Schematic diagram of XY4-C7 administration and HCoV-OC43 challenge. (g) \textit{In vivo} efficacy of XY4-C7 (1 mg/kg) against HCoV-OC43 infection in newborn mice. Viral RNA expression level in the brain tissue of mice in each group on the 4th day post-infection was detected.
Based on its potent ability to invade the central nervous system (CNS) and affect the function of particular nuclei or neural circuits, SARS-CoV-2 causes a variety of severe neurological symptoms and complications, including acute stroke, hyposmia, Guillain-Barré syndrome, and encephalitis. As such, we employed the PAMPA-BBB assay to evaluate the potential of XY4-C7 to cross the BBB and predict its ability to prevent SARS-CoV-2 from invading the CNS, thus controlling these symptoms and complications. Verapamil, the positive control, could easily cross the BBB with a $P_{\text{app}}$ value of $148 \times 10^{-6}$ cm/s at a concentration of 50 µM (Figure 22c). In contrast, Theophylline, the negative control, had a low $P_{\text{app}}$ value of $4 \times 10^{-6}$ cm/s, even at a concentration of 250 µM, and could barely cross the BBB (Figure 22c). Like Verapamil, XY4-C7 (50 µM) easily passed through the BBB with a $P_{\text{app}}$ value of $132 \times 10^{-6}$ cm/s, which may help to explain why it demonstrated potent protection of mouse brain against HCoV-OC43 infection in the HCoV-OC43-infected mouse model (Figure 21g and 22c). Therefore, we can predict that XY4-C7 will have very promising potential to control the SARS-CoV-2 in CNS.

### 3.2.8 XY4-C7 has favorable pharmacokinetic (PK) profiles with much longer half-life and very promising oral bioavailability in mouse

To determine the oral absorption and in vivo stability of XY4-C7, we performed PK studies in mice via intraperitoneal (IP) and oral administration (OP) of XY4-C7 at 30 mg/kg over 48 h. In IP administration, the average maximum blood concentration ($C_{\text{max}}$) of 408,769 µg/L was achieved within 5 hours, while in OP administration, $C_{\text{max}}$ of 114,140 µg/L was reached after 4 hours (Figure 22d and 22e). This suggests that effective blood exposure was approximately 350-fold and 97-fold higher than the IC$_{50}$ of XY4-C7 in IP and OP administration, respectively. Additionally, the effective period of both administrations is over 24 h, and the plasma concentration decayed with a
longer half-life \( (t_{1/2}) \) of 9.4 h in IP administration and a significantly longer \( t_{1/2} \) of 28.9 h in OP administration (Figure 22d and 22e). Most importantly, **XY4-C7** again displayed high oral bioavailability (F%) of 28%, indicating that it possesses the promising potential for use as an orally delivered drug (Figure 22d and 22e).

### 3.3 Discussion

To combat the SARS-CoV-2 pandemic, as well as emerging and re-emerging HCoVs in the future, particularly among the unvaccinated segment of the global population and rising concerns about drug resistance to various variants, it is urgently necessary to develop long-acting oral drugs with broad-spectrum activity across HCoVs. Bioactive peptides like EK1, EK1C4, and [SARS\(_{HRC}\)-PEG\(_4\)]\(_2\)-chol, which were designed as pan-CoV fusion inhibitors, have already shown potent inhibitory activity against SARS-CoV-2.\(^7,16,18\) However, their use as long-acting oral drugs is challenging owing to their limited bioavailability and biostability by the lack of a native peptide backbone. Peptidomimetics, which are designed to mimic the structure and function of bioactive peptides and proteins, have shown remarkable applications in protein surface mimicry and recognition, modulation of PPIs, and catalysis. Recently, we created and applied sulfonyl-\(\gamma\)-AApeptides as a new helical framework to design protein helical domain mimetics and modulate a variety of medicinally relevant PPIs, including VEGF/VEGFR, p53/MDM2, GLP-1, BCL9/\(\beta\)-catenin, and others. Most of these were constructed from L-sulfonyl-AApeptide building blocks, which have left-handed helical conformations in contrast to the right-handedness of \(\alpha\)-peptides. In this article, we reported D-sulfonyl-\(\gamma\)-AApeptides-based right-handed helical foldamers, which were anticipated to display right-handed helical conformation more in line with that of \(\alpha\)-helix, to mimic the HR2 peptide in the fusion core and thus prevent the SARS-CoV-2 fusion process.
The current design was based on the crystal structure of the HR2 fusion core in a complex with HR1 trimer. Most critical residues of the HR2 helix in the fusion core, including Ile1179, Ile1183, Leu1186, Val1189, Leu1193, and Leu1197, are involved in binding HR1 trimer. Therefore, some sulfonyl-γ-AApeptides were designed based on the helical structures to reproduce these hydrophobic functionalities using the chiral side chains at 1a, 3a, 5a, 7a, 9a, and 11a, respectively, on the same face of sulfonyl-γ-AApeptide foldamers. Hydrophilic groups were included on the other two faces of our foldamers, which are not involved in the interaction with the HR1 region in the fusion core, to improve the helical stability and solubility. Based on these design principles, we have shown that some sulfonyl-γ-AApeptides exhibit excellent binding affinity and strong interaction with the hydrophobic surface of the HR1 peptide. These results demonstrated that these sulfonyl-γ-AApeptides successfully mimicked the HR2 peptide in the fusion core by interacting with the HR1 trimer. Following validation by PsV infection assay, the lead sequence XY4 was chosen for further optimization. Since lipidation is known to improve the efficacy of fusion inhibitors, we added two different linkers, the rigid and the flexible one, along with various PEG lengths with Chol. We found that XY4-C7 retained its binding affinity and interacting capability with the HR1 peptide and exhibited highly potent activity in the authentic SARS-CoV-2 infection assay with IC₅₀ of 0.24 μM and SI of 62. Moreover, XY4-C7 is also highly effective against infection by authentic SARS-CoV-2 Delta variant, and the pseudotyped SARS-CoV, MERS-CoV, and HCoV-NL63, as well as SARSr-CoV WIV1 from the bat. Following the \textit{in vitro} test, we found that intranasally applied XY4-C7 to newborn HCoV-OC43 mice potently inhibited its infection \textit{in vivo}. Most importantly, \textit{in vitro} and \textit{in vivo} PK studies further proved that XY4-C7 was highly resistant to proteolytic degradation and had an extremely long half-life and very promising oral bioavailability.
3.4 Conclusion

In conclusion, we have identified several unnatural helical foldameric mimetics of the HR2 peptide in the fusion core in the S2 subunit of the SARS-CoV-2 S protein. Upon validation, we have found that the lead compound, XY4-C7, is a highly potent pan-CoV fusion inhibitor against infection by SARS-CoV-2 and its Delta variant, and several HCoVs, including SARS-CoV, MERS-CoV, HCoV-NL63 and HCoV-OC43, as well as bat SARSr-CoV WIV1. Additionally, it showed outstanding PK properties in both PAMPAs and PK tests (both oral and injection administrations). Therefore, it is reasonable to assume that XY4-C7 can be further developed as a novel orally applicable anti-HCoV drug and combining XY4-C7 with other available COVID-19 therapeutics with different mechanisms of action may have a synergistic antiviral effect, resulting in a new cocktail for the treatment of infection of SARS-CoV-2 and other HCoVs. Overall, we believe that this work can be broadened to develop different antiviral agents using sulfonyl-γ-AApeptides, as well as utilized to modulate thousands of other PPIs.

3.5 Materials and methods

3.5.1 Synthesis of sulfonyl-γ-AApeptide building blocks

3.5.1.1 General information

All chemicals and solvents were purchased and directly used without any purification from Fisher Scientific, Sigma-Aldrich, or Oakwood. Fmoc protected amino acids were purchased from Chem-Impex International. ¹H-NMR at 600 MHz and ¹³C-NMR at 150 MHz using TMS as the internal standard were acquired from Inova 600 MHz NMR spectrometer. The mass of each building block was determined by high-resolution mass spectrometry detected by High-resolution MS (HRMS) on Agilent 6540 LC/QTOF.
Figure 22 Evaluation of stability, membrane passive permeability, oral bioavailability, and PK profiles of XY4-C7 in a mouse model

(a) Stability studies of HR2 peptide mimics. LC/MS/MS of the indicated control and XY4-C7 incubated in the presence of pronase or human sera, respectively, for 24 h. (b) PAMPA-GIT for standards, and XY4-C7 at different pH conditions. (c) PAMPA-BBB for standards and XY4-C7 at pH 7.4. (d) Time-concentration plot of XY4-C7 in PK study. Plasma concentration and time curve following intraperitoneal (IP) (red) and oral administration (OP) (blue) administration of 30 mg/kg XY4-C7 in C57BL/6 mice (data indicated are means ± SD, n = 3). (e) Pharmacokinetics parameters of XY4-C7 over 48 h in mice.

<table>
<thead>
<tr>
<th></th>
<th>XY4-C7 (OP)</th>
<th>XY4-C7 (IP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AUC_{0-\infty}$ (μg/L*h)</td>
<td>3,556,398±3,563,936</td>
<td>6,113,501±2,930,203</td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>4</td>
<td>5.33±2.31</td>
</tr>
<tr>
<td>$C_{max}$ (μg/L)</td>
<td>114,140±70,985</td>
<td>408,769±243,692</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>28.87±20.81</td>
<td>9.39±1.82</td>
</tr>
<tr>
<td>$F%$</td>
<td>28%</td>
<td></td>
</tr>
</tbody>
</table>

*Each group had three mice, $T_{1/2}$ was calculated as the terminal elimination half-time.
3.5.1.2 Synthesis

The sulfonyl-γ-AAPeptide building blocks were synthesized based on a previous report and Fmoc-protected amino acids were used as the initial starting materials. The building blocks were synthesized using one of the following three routes, depending on the protective groups used (Figure 23). Building blocks BB1-BB4 were synthesized by route A. BB5 and BB6 were synthesized by route B and BB7-BB11 were synthesized by route C.

3.5.1.3 Characterization of sulfonyl-γ-AAPeptide building blocks

(S)-N-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(tert-butoxy)propyl)-N-(methylsulfonyl)glycine (BB7). $^1$H NMR (600 MHz, Chloroform-$d$) $\delta$ 7.76 (d, $J = 7.5$ Hz, 2H), 7.60 (dd, $J = 7.6, 4.4$ Hz, 2H), 7.40 (t, $J = 7.5$ Hz, 2H), 7.34 – 7.30 (m, 2H), 4.42 (dd, $J = 10.6, 7.2$ Hz, 1H), 4.36 – 4.30 (m, 2H), 4.23 (t, $J = 7.2$ Hz, 1H), 4.15 – 4.10 (m, 1H), 3.95 (d, $J = 10.4, 7.2$ Hz, 1H), 4.41 (dd, $J = 10.4, 7.2$ Hz, 1H), 4.31 (d, $J = 20.5$ Hz, 2H), 4.23 (t, $J = 7.4$ Hz, 1H), 3.94 (s, 1H), 3.73 – 3.55 (m, 3H), 3.37 (d, $J = 11.6$ Hz, 1H), 3.31 (s, 2H), 3.25 – 3.19 (m, 1H), 1.49 (d, $J = 6.3$ Hz, 3H), 1.44 (d, $J = 3.1$ Hz, 9H). $^{13}$C NMR (150 MHz, Chloroform-$d$) $\delta$ 172.82, 156.66, 143.74, 141.29, 127.75, 127.14, 125.23, 120.01, 73.76, 67.19, 60.96, 49.13, 48.67, 48.16, 47.09, 39.59, 27.43. HRMS (ESI) ($[M+H]^+$) Calcd. for C$_{25}$H$_{32}$N$_2$O$_7$S: 505.1930, found: 505.1927.

(R)-N-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)propyl)-N-((2-((tert-butoxycarbonyl)amino)ethyl)sulfonyl)glycine (BB8). $^1$H NMR (600 MHz, Chloroform-$d$) $\delta$ 7.76 (d, $J = 7.6$ Hz, 2H), 7.61 (t, $J = 6.9$ Hz, 2H), 7.40 (t, $J = 7.5$ Hz, 2H), 7.32 (t, $J = 7.5$ Hz, 2H), 7.26 (s, 1H), 4.41 (dd, $J = 10.4, 7.2$ Hz, 1H), 4.31 (d, $J = 20.5$ Hz, 2H), 4.23 (t, $J = 7.4$ Hz, 1H), 3.94 (s, 1H), 3.73 – 3.55 (m, 3H), 3.37 (d, $J = 11.6$ Hz, 1H), 3.31 (s, 2H), 3.25 – 3.19 (m, 1H), 1.49 (d, $J = 6.3$ Hz, 3H), 1.44 (d, $J = 3.1$ Hz, 9H). $^{13}$C NMR (150 MHz, Chloroform-$d$) $\delta$ 171.99, 156.46, 143.80,
141.28, 127.71, 127.14, 125.33, 125.23, 119.97, 80.66, 67.05, 60.53, 52.08, 47.10, 44.41, 35.33, 28.36, 21.10, 18.59. HRMS (ESI) ([M+H]+) Calcd. for C_{27}H_{35}N_{3}O_{8}S: 562.2145, found: 562.2138.

**Figure 23 General routes of synthesis of sulfonyl-γ-AApeptide building blocks**
(R)-N-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-methylpentyl)-N-((2-((tert-butoxy carbonyl)amino)ethyl)sulfonyl)glycine (BB10). $^1$H NMR (600 MHz, Chloroform-$d$) $\delta$ 7.75 (dd, $J = 7.6$, 2.3 Hz, 2H), 7.61 (dd, $J = 11.2$, 7.3 Hz, 2H), 7.39 (t, $J = 7.4$ Hz, 2H), 7.31 (t, $J = 7.5$ Hz, 2H), 7.26 (s, 1H), 4.94 (dd, $J = 26.6$, 9.4 Hz, 1H), 4.45 (dd, $J = 10.6$, 6.9 Hz, 1H), 4.36 – 4.21 (m, 3H), 3.92 (s, 1H), 3.68 – 3.56 (m, 2H), 3.33 (t, $J = 7.7$ Hz, 1H), 3.28 (td, $J = 13.5$, 11.0, 7.3 Hz, 2H), 3.21 (d, $J = 17.9$ Hz, 1H), 1.67 (m, 1H), 1.46 (d, $J = 34.3$ Hz, 1H), 0.92 (t, $J = 7.0$ Hz, 6H).

$^{13}$C NMR (150 MHz, Chloroform-$d$) $\delta$ 172.09, 156.81, 143.74, 141.30, 127.68, 127.13, 125.22, 124.65, 119.94, 80.68, 66.95, 60.58, 52.90, 51.61, 47.21, 41.84, 35.34, 28.36, 24.81, 23.25, 21.96.

HRMS (ESI) ([M+H]$^+$) Calcd. for C$_{30}$H$_{41}$N$_3$O$_8$S: 604.2614, found: 604.2599.

Other building blocks are characterized in our previous works$^{42}$.

![Structure of sulfonyl-$\gamma$-AApeptide building blocks](image)
3.5.2 *Synthesis of Chol-PEGn-bromoacetate*

The Chol-PEGn were synthesized according to the previous report\(^{46}\) and the cholesterol was used as starting material. Chol-PEG4, Chol-PEG8, Chol-PEG12, and Chol-PEG24 were synthesized using the following routes (Figure 25). Chol-PEGn-bromoacetate were also synthesized based on the previous report\(^{47}\) and using the following routes. Briefly, a mixture of Chol-PEG4, Chol-PEG8, Chol-PEG12, or Chol-PEG24 with bromoacetic acid was dissolved in CH\(_2\)Cl\(_2\). Then 2 eq of N,N-diisopropylcarbodiimide (DIPEA) and 0.01 eq of 4-dimethylaminopyridine (DMAP) were added. The solution was left stirring at room temperature for 48 h and analyzed by TLC. Afterward, the solvent was removed under vacuum, and the crude was purified by flash column chromatography on silica gel as a white solid.

3.5.3 *Preparation of sulfonyl-\(\gamma\)-AA peptide sequences mimicking the HR2 peptide on the fusion core*

3.5.3.1 *General information*

Solid phase synthesis was carried out in the peptide synthesis vessels on the Burrell Wrist-Action shaker. Rink amide-MBHA resin (0.6 mmol/g) was used for the synthesis of sulfonyl-\(\gamma\)-AA peptides. All peptides were analyzed and purified using a method that involved a 5-100% linear gradient of solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in H\(_2\)O) over a 50-minute period, followed by 100% solvent B over a 15-minute period, on a Waters Breeze 2 HPLC system equipped with both analytic column (1 mL/min) and preparative column (16 mL/min). A Labconco freeze-drying machine was used to lyophilize purified sequences. The mass of each sulfonyl-\(\gamma\)-AA peptides sequence was confirmed with MALDI on Applied Biosystems 4700 Proteomics Analyzer.
3.5.3.2 Synthesis

3.5.3.2.1 Synthesis of sulfonyl-$\gamma$-AApeptides

Sulfonyl-$\gamma$-AA peptide sequences were synthesized based on our previous reports\(^6\) and followed the route in Figure 3. 10. Solid phase synthesis was started from 100 mg Rink Amide-
MBHA resin (0.6 mmol/g) under room temperature at atmospheric pressure. The resin was first swelled by soaking in DMF for 10 min, then the Fmoc group was deprotected by shaking in 20% piperidine/DMF (15 min × 2), and then the resin was washed three times with DCM and three times with DMF. The coupling reaction was finished by adding a premixed solution of the Fmoc-protected regular amino acid/sulfonyl-γ-AApeptide building block (2 eq), HOBt (4 eq), and DIC (4 eq) in 3 mL DMF to the resin and shaking for 4 h. The resin was treated with 20% piperidine/DMF solution (15 min × 2) after being washed with DCM and DMF. Following the same coupling procedure, another Fmoc-protected regular amino acid/sulfonyl-γ-AApeptide building block was attached to the resin. The reaction cycles were carried out repeatedly until the desired sulfonyl-γ-AApeptides were synthesized. After the N-terminus of the sulfonyl-γ-AApeptide sequences was acetylated by acetic anhydride (1 mL) in pyridine (2 mL) for 15 min, the desired sequences were cleaved from the resin by treating with 1:1 TFA/DCM solution (4 mL, 2h). The resin was washed with DCM three times after the cleavage solution was collected. The cleavage solution and the wash solutions were combined, and the resulting crude product was vacuum dried. The crude sequences were analyzed and purified on a Waters HPLC system. All the HR2 fusion core mimic sulfonyl-γ-AApeptides were obtained at > 95% purity after prep-HPLC purification.

3.5.3.2.2 Synthesis of Chol-PEGn-sulfonyl-γ-AApeptides

The synthesis of Chol-PEGn-sulfonyl-γ-AApeptides was based on the previous reported. Briefly, Chol-PEGn-bromoacetate (1 eq) in CH3CN was added to a solution of purified sulfonyl-γ-AApeptide sequence in 30 mM NH4HCO3 buffer. The mixture was stirred at room temperature for 4 h. Acetic acid was subsequently added dropwise to pH 5 under ice cooling. Chol-PEGn-
sulfonyl-γ-AApeptides were purified by preparative reverse phase HPLC to afford the compound as a white powder after lyophilization.

Figure 26 General routes of synthesis of sulfonyl-γ-AApeptides (a), and Chol-PEGn-sulfonyl-γ-AApeptides (b)

3.5.3.3 Characterization of sulfonyl-γ-AApeptides and Chol-PEGn-sulfonyl-γ-AApeptides

XY1

Chemical Formula: C_{104}H_{211}N_{31}O_{40}S_{11}

Theoretical Mol. Wt: 2888.6690

Observed (MALDI-TOF): 2889.5367 (M+H⁺)

Purity: 98.62%
**FITC-XY1**

Chemical Formula: C\textsubscript{126}H\textsubscript{227}N\textsubscript{33}O\textsubscript{45}S\textsubscript{12}  

Theoretical Mol. Wt: 3309.1080  

Observed (MALDI-TOF): 3332.1123 (M+Na\textsuperscript{+})  

Purity: 98.02%

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**XY2**

Chemical Formula: C\textsubscript{107}H\textsubscript{217}N\textsubscript{31}O\textsubscript{40}S\textsubscript{11}

81
Theoretical Mol. Wt: 2930.7500

Observed (MALDI-TOF): 2931.4500 (M+H⁺)

Purity: 99.33%

FITC-XY2

Chemical Formula: C₁₂₉H₂₃₃N₃₃O₄₅S₁₂

Theoretical Mol. Wt: 3351.1890

Observed (MALDI-TOF): 3391.1580 (M+K⁺)

Purity: 99.16%
XY3

Chemical Formula: C_{107}H_{217}N_{31}O_{40}S_{11}

Theoretical Mol. Wt: 2930.7500

Observed (MALDI-TOF): 2931.6553 (M+H^+)

Purity: 99.58%

FITC-XY3

Chemical Formula: C_{129}H_{233}N_{33}O_{45}S_{12}

Theoretical Mol. Wt: 3351.1890

Observed (MALDI-TOF): 3352.2220 (M+H^+)

Purity: 99.90%
XY4

Chemical Formula: C_{106}H_{214}N_{30}O_{40}S_{11}

Theoretical Mol. Wt: 2901.7080

Observed (MALDI-TOF): 2940.7300 (M+K⁺)

Purity: 99.98%

FITC-XY4
Chemical Formula: $\text{C}_{128}\text{H}_{230}\text{N}_{32}\text{O}_{45}\text{S}_{12}$

Theoretical Mol. Wt: 3322.1470

Observed (MALDI-TOF): 3323.1560 (M+H$^+$)

Purity: 99.88%

---

XY5

Chemical Formula: $\text{C}_{113}\text{H}_{234}\text{N}_{32}\text{O}_{36}\text{S}_{11}$

Theoretical Mol. Wt: 2969.9630

Observed (MALDI-TOF): 2970.8878 (M+H$^+$)

Purity: 99.19%
XY6

Chemical Formula: C\textsubscript{101}H\textsubscript{211}N\textsubscript{31}O\textsubscript{33}S\textsubscript{10}

Theoretical Mol. Wt: 2708.5830

Observed (MALDI-TOF): 2709.5950 (M\textsuperscript{+H\textsuperscript{+}})

Purity: 99.50%

XY7

Chemical Formula: C\textsubscript{101}H\textsubscript{210}N\textsubscript{30}O\textsubscript{33}S\textsubscript{10}

Theoretical Mol. Wt: 2693.5680

Observed (MALDI-TOF): 2694.5655 (M\textsuperscript{+H\textsuperscript{+}})

Purity: 99.35%
Chemical Formula: C_{105}H_{221}N_{31}O_{31}S_{10}

Theoretical Mol. Wt: 2734.7090

Observed (MALDI-TOF): 2735.7111 (M+H^+)

Purity: 99.75%

XY4-C1
Chemical Formula: $C_{158}H_{303}N_{33}O_{49}S_{12}$

Theoretical Mol. Wt: 3830.8881

Observed (MALDI-TOF): 3853.4200 (M+Na$^+$)

Purity: 98.58%

---

Chemical Formula: $C_{166}H_{319}N_{33}O_{53}S_{12}$

Theoretical Mol. Wt: 4006.9930

Observed (MALDI-TOF): 4029.7800 (M+Na$^+$)

Purity: 99.00%
**XY4-C3**

Chemical Formula: \( \text{C}_{174}\text{H}_{335}\text{N}_{33}\text{O}_{57}\text{S}_{12} \)

Theoretical Mol. Wt: 4186.4880

Observed (MALDI-TOF): 4209.9200 (M+Na\(^+\))

Purity: 99.32%

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**XY4-C4**

Chemical Formula: \( \text{C}_{158}\text{H}_{305}\text{N}_{35}\text{O}_{55}\text{S}_{14} \)

Theoretical Mol. Wt: 4024.2080

Observed (MALDI-TOF): 4025.4000 (M+H\(^+\))

Purity: 98.79%
**XY4-C5**

Chemical Formula: C_{166}H_{321}N_{35}O_{59}S_{14}

Theoretical Mol. Wt: 4196.9284

Observed (MALDI-TOF): 4197.2000 (M+H⁺)

Purity: 98.11%

**XY4-C6**

Chemical Formula: C_{174}H_{337}N_{35}O_{63}S_{14}
Theoretical Mol. Wt: 4376.6320

Observed (MALDI-TOF): 4377.0612 (M+H+)

Purity: 98.50%

XY4-C7

Chemical Formula: C\textsubscript{198}H\textsubscript{385}N\textsubscript{35}O\textsubscript{75}S\textsubscript{14}

Theoretical Mol. Wt: 4905.2680

Observed (MALDI-TOF): 4906.1606 (M+H+)

Purity: 99.45%
FITC-XY4-C7

Chemical Formula: C220H401N37O80S15

Theoretical Mol. Wt: 5325.7070

Observed (MALDI-TOF): 5358.9414

Purity: 99.36%

3.5.4 Fluorescence polarization assay

Proteins (0-2 μM) in PBS were treated with 50 nM FITC were treated with 50 nM FITC-labeled γ-AApeptides. Fluorescence anisotropy values were plotted as a function of protein concentration to determine the dissociation onstants (Kd), and the graphs were then fitted to the following equation.

\[
y = \frac{[FP_{\text{min}} + (FP_{\text{max}} - FP_{\text{min}})]}{(K_d + L_{st} + x) - \sqrt{(K_d + L_{st} + x)^2 - 4L_{st} x}}
\]

L_{st} and x refer to the concentration of the peptide and protein, respectively. The experiments were conducted in triplicate and repeated three times.
3.5.5 Circular dichroism spectroscopy

With the use of an Aviv 215 spectrometer in a 1 mm path quartz cuvette, CD spectra of samples were obtained in PBS at 100 μM concentrations. An average of 3 scans for each sample run was taken thrice and averaged. The PBS sample that was used as blank was evaluated the same method and subtracted from average sample readings. The equation below was used to calculate molar ellipticity $[\theta]$.

$$[\theta] = \frac{\theta_{\text{obs}}}{(n \times l \times c \times 10)}$$

$\theta_{\text{obs}}$ = measured ellipticity in millidegrees

n = number of side chains

l = path length in centimeter (0.1 cm)

c = concentration of samples (M)

CD spectra of mechanism were recorded by previous method. The HR1 peptide (911-987, NovoPep Limited), the HR2 peptide (1175-1201, synthesized) and XY4-C7 were dissolved in PBS buffer at a final concentration of 40 μM. Briefly, HR1 peptides were mixed with HR2 helices at 37 °C for 1 h. After that, added XY4-C7 to further incubated for 30 min. The CD wave scans were measured by an Aviv 215 spectrometer from 190 to 260 nm.

The HR1 peptide (911-987):

TQNVLYENQKLIANQFNSAIGKIQDLSSTASALGKLQDVVNQNAQALNTLVKQLSSNF

GAISSVLNDILSDLDKVE

The HR2 peptide (1175-1201):

SVVNIQKEIDRLNEVAKNLNESLIDLQ
3.5.6  **PAMPA-BBB assay**

The PAMPA-BBB assay produce was developed by pION that followed by previous reported. The TECAN Freedom EVO150 robot carried out each step of the liquid handling process, and Pion PAMPA Evolution software was used to analyze the results. BBB PAMPA consisted of Stirwell\textsuperscript{TM}PAMPA Sandwich plate preloaded with magnetic stirring disks, brain the sink buffer (BSB), and lipid solution (BBB-1). 200 μL of BSB (pH 7.4) was added to the acceptor well after 4 μL of lipid solution had been put there. 180 μL of diluted \textit{XY4-C7} (50-250 μM in system buffer at pH 7.4 from a 10 mM DMSO stock solution) was then added to the donor well. The PAMPA sandwich plate was assembled, placed on the Gut-Box\textsuperscript{TM} and stirred with 60 μm Aqueous Boundary Layer (ABL) settings for 1 h incubation. The distribution of compounds in the donor and acceptor buffers (150 μL aliquot) was determined using the TECAC S20Infinnite M-1000 Pro microplate reader to measure UV spectra from 250 to 498 nm. Permeability (P\textsubscript{app}, 10^{-6} cm/s) of each compound was calculated by Pion PAMPA evolution software. The assay was performed in triplicate.

3.5.7  **PAMPA-GIT assay**

The PAMPA-GIT assay was also carried out utilizing a pION-developed technique. All liquid handling procedures were performed using the TECAC Freedom EVO 150 robot, and the data was evaluated using pION’s PAMPA Evolution software. The acceptor sink buffer (ASB), GIT-0 Lipid solution, and the Stirwell\textsuperscript{TM} PAMPA sandwich plate preloaded with magnetic disks are all included in the pION’s GIT PAMPA. In the acceptor well, 4 μL of lipid were transferred, followed by 200 μL of ASB (pH 7.4). The donor wells were then filled with 180 μL of diluted test compound (50-250 M in system buffer at pH 5.0, 6.2, and 7.4 from a 10 mM DMSO solution).
The PAMPA sandwich plate was put together and positioned on the Gur-BoxTM. It was then agitated for 30 min at 40 μm Aqueous Boundary Layer (ABL) settings. The distribution of the compounds in the donor and acceptor buffers (150 μL aliquot) was determined by UV spectra measurement from 250 to 498 nm using the TECAN Infinite M-1000 Pro microplate reader. Then the Permeability (P_{app}, \, 10^{-6} \text{cm/s}) of each compound was calculated by Pion PAMPA evolution software. The assay was performed in triplicate.

### 3.5.8 ITC assay

The interaction between XY4-C7 and the HR1 peptide was determined using an ITC microcalorimeter instrument. In brief, the HR1 peptide was dissolved in ACN/H2O to 10 μM before being injected into the chamber containing 100 μM of XY4-C7. Titration was carried out at a constant stirring speed of 220 rpm and temperature of 23 °C. Twenty-five injections were automatically performed with an injection interval of 180 sec. After that, iTC-200 software was used to analyze the data.

### 3.5.9 Enzyme stability assay

XY4-C7 or the HR2 peptide (0.1 mg/mL) were incubated with 0.1 mg/mL pronase in 100 mM ammonium bicarbonate buffer (pH 7.8) at 37 °C for 24 h. Then, a speed vacuum was used to remove the water and ammonium bicarbonate from the reaction mixtures. The residues were dissolved in 100 μL ACN and analyzed by LC/MS/MS.
3.5.10 Serum stability assay

The serum stability of XY4-C7 or the HR2 peptide was determined in 50% (v/v) aqueous serum from male AB plasma.\textsuperscript{14} XY4-C7 or the HR2 peptide was dissolved in 30 μL DMSO, to which was added 970 μL of 50% aqueous pooled serum to make 1 mg/mL solution. The solution was incubated at 37 °C for 24 h. Subsequently, 100 μL of ACN was added to 100 μL of serum incubating solution on ice for 20 min, which was then centrifuged at 4 °C for 15 min. The supernatant should have a concentration at 0.5 mg/mL. A final concentration of 0.1 mg/mL can be achieved by diluting 50 μL of supernatant with 200 μL H₂O. The vials containing diluted supernatants were then used for the LC/MS/MS analysis.

3.5.11 In vitro anti-virus assays

3.5.11.1 Inhibition of pseudovirus infection

The inhibitory activity of sulfonyl-γ-AApeptides was tested according to our previous publications.\textsuperscript{22,23} In our lab, we kept plasmids encoding the S protein of coronaviruses such as SARS-CoV-2, SARS-CoV, MERS-CoV, HCoV-NL63 and SARSr-CoV WIV1, luciferase reporter vectors (pNL4-3, Luc.R-E-). The pcDNA3.1-SARS-CoV-2-S and pNL4-3. Luc.R-E- were co-transfected into HEK-293T cells for the packaging of pseudoviruses using Vigofect transfection reagent, and the supernatants were then replaced with fresh media containing 10% FBS after eight hours. After 48 hours, the pseudovirus-containing supernatants were gathered, filtered using a 0.45 μm filter, and then stocked at -80 °C. Target cells were seeded at 8000 per well in a 96-well plate and grown at 37 °C for 12 h to ascertain a compound’s inhibitory effect. The compound was diluted with DMEM without PBS, and then the same volume of pseudoviruses was added. The mixture (100 μL) was then added to the target cells and allowed to sit there for 30
minutes. The mixture was changed out to fresh medium after 12 h. After 48 h, the cells were lysed with cell lysis buffer, and the Luciferase Assay System was used to find luciferase activity (Promega, Madison, WI, USA).

3.5.11.2 Inhibition of authentic coronavirus infection

As previously reported, the SARS-CoV-2 inhibition assay was carried out in a biosafety level 3 laboratory (BSL-3). The inhibitory activity of XY4-C7 was evaluated against authentic viruses (SARS-CoV-2/SH01/human/2020/CHN). XY4-C7 was serially diluted with DMEM without PBS. The diluted XY4-C7 was combined with 100 TCID50 of the virus. The mixtures were transferred to target cells (Caco2 cells or Vetro-E6 cells) after incubation for 30 minutes. The supernatants were collected after 48 h and the viral RNA load was tested as previously reported (reference). Briefly, the viral RNA was extracted with an RNA extraction kit (Transgene, China). Then the N gene of SARS-CoV-2 was evaluated by real-time RT-PCR. The sequence of primer and probe follows:

Forward: GGGGAACCTTCTCCTGCTAGAAT;
Reverse: CAGACATTTTGCTCTCAAGCTG
Probe: 5′-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3′

3.5.11.3 Inhibition of S protein-mediated cell-cell fusion

The cell-cell fusion assay was performed as in previous reports. PAAV-IRES-EGFP S was transfected to HEK-293T cells to obtain effector cells expressing the S protein of SARS-CoV-2 and GFP. After that, serially diluted XY4-C7 was mixed with effector cells (HEK-293T), and
the mixture was transferred to target cells (Caco-2 cells). After incubation for 2-4 h, fused cells were counted, and the fusion rate was calculated to determine inhibitory activity.

### 3.5.11.4 Cytotoxicity assay

The cytotoxicity of XY4-C7 to Caco-2 cells was tested as previously described.\textsuperscript{22,23} Briefly, we added the serially diluted compounds to the target cells (Caco-2 cells). The medium was changed to a fresh medium after culture at 37 °C for 12 h. The supernatant was removed after 48 h later and cell viability was analyzed with Cell Counting Kit (CCK-8; Dojindo, Kumamoto, Japan). 100 μL Cell Counting Kit-8 solution was added to each well in a 96-well plate and followed by an additional incubation for 2 h. The absorbance was measured at 450 nm.

### 3.5.12 In vivo assays

#### 3.5.12.1 Evaluation of the in vivo protective activity of XY4-C7

The protective effect of XY4-C7 against HCoV-OC43 in vivo was performed according to a previous study.\textsuperscript{22,23} The Institutional Laboratory Animal Care and Use Committee at Fudan University approved the use of animals in this study (Approval number: 20200821-002). Newborn mice infected with HCoV-OC43 were established as previously reported.\textsuperscript{22,23} Pregnant Balb/c mice (18 days) were separated into three groups after giving birth. Seven baby mice were split among each group. XY4-C7 was intranasally administered at a dose of 1 mg/kg into mice in the preventive and therapeutic groups before or after the HCoV-OC43 challenge. Dissection of the newborn mice was done on the fourth post-infection day. The relative viral RNA expression level in the brain was evaluated through RT-PCR and calculated as $2^{(-\Delta\Delta Ct)}$. The HCoV-OC43 RNA level was
adjusted with the mouse housekeeping gene GAPDH. The primer of HCoV-OC43 and GAPDHA follows:

OC43-S-Forward: GACACCGGTCCCTCCTCTAT;
OC43-S-Reverse: ACACTTCCCTTCAGTGCCAT;
GDPAH-Forward: TGCTGTCCCTGTATGCCTCTG;
GDPAH-Reverse: TTGATGTACGCACGATTTCC.

3.5.12.2 Mouse pharmacokinetic studies

XY4-C7 was administered to C57BL/6 mice in two different studies at doses of 30 mg/kg, 150 μL, either OP or IP. Following administration, 100 μL blood samples were taken at 10 min, 20 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, and 48 h (n = 3 per time point; each mouse was used for three time points, making a total of 9 mice utilized for either OP or IP, making a total of 18 mice.). After drug administration, 100 μL of blood were collected into 1.5 mL Eppendorf tubes containing 30 μL disodium ED (0.5 M, pH 8.0) and kept on ice until plasma collection (< 30 min), followed by centrifugation at 4000 rpm/min for 10 min at 4 °C. The supernatants were collected and stored at -80 °C for future analysis. 50 μL of serum samples were mixed with 15 μL of glacial acetic acid and 135 μL of acetonitrile. The samples were centrifuged at 10,000 rpm and 4 °C for 15 min after resting on ice for 15 min. Vials containing clarified supernatants were used for the LC/MS/MS analysis. PK parameters were obtained using PKSolver.
3.6 $^1$H and $^{13}$C NMR spectra of D-sulfonyl-γ-AA peptide building blocks

$^1$H NMR of BB7

$^{13}$C NMR of BB7
3.7 References


Chapter 4  Rational Design of Sulfonyl-γ-AAApeptides as Highly Potent HIV-1 Fusion Inhibitors with Broad-spectrum Activity

Note to Reader

Contents of this chapter are currently in review by *J. Med. Chem.*

4.1 Introduction

Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus (HIV), continues to be one of the most dangerous diseases worldwide, resulting in 1.5 million new infections and almost 1 million deaths in 2021.¹ With the successful application of highly active antiretroviral therapy (HAART) all over the world, the morbidity and mortality associated with AIDS have profoundly decreased.² So far, the combination antiretroviral therapy regimen consists of several classes of antiviral therapeutics, including nucleoside/non-nucleoside reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors, coreceptor inhibitors and integrase inhibitors.³ As none of the strategies is able to completely eradicate the virus, and most patients must be medicated for several years, or even lifelong, resulting in unwanted side effects and drug resistance.⁴ As a result, developing novel antiviral drugs is the most effective way to elevate the efficiency of HAART.

Similar to the spike protein of SARS-CoV-2,⁵⁻⁷ the HIV type 1 (HIV-1) envelope (Env) protein has been an attractive target for the development of anti-HIV-1 agents. HIV-1 Env spike is a type I membrane protein sitting in the viral membrane that initiates infection by attaching the
virion to a susceptible cell and then induces the fusion of viral and cell membranes.\textsuperscript{8} Env spike is a trimer composed of non-covalently associated heterodimers of glycoprotein 120 (gp120) that interacts with cellular receptors CD4 and chemokine coreceptor CCR5 or CXCR4, as well as gp41, the transmembrane component that mediates fusion between viral and target membranes (Figure 27A and Figure 27B).\textsuperscript{9} The subunit gp120 binding with primary receptors triggers a series of conformational changes in gp41.\textsuperscript{10} The N-terminal fusion peptide (FP) of gp41 inserts into the target cell membrane, forming an extended, prehairpin trimer intermediate, after which the N-terminal heptad repeat (NHR) domain of gp41 interacts with the C-terminal heptad repeat (CHR) domain to form a hairpin-like 6-helix bundle (6-HB) structure, pulling the viral particle and host cell into proximity for fusion (Figure 27B and Figure 27C).\textsuperscript{11-13} Within the 6-helix bundle structure, we highlight the formation of a deep hydrophobic pocket located in the C-terminal portion of NHR helices, which is penetrated by three hydrophobic residues (Trp628, Trp631, and Ile635) from the pocket-binding domain (PBD) of the CHR helix (Figure 27D).\textsuperscript{14} This deep pocket stabilizes the 6-HB core and offers a desirable targeting site for the development of new therapeutic anti-HIV-1 agents.\textsuperscript{15} Enfuvirtide (T20), a 36-residue α-peptide derived from the CHR region and targeted to the NHR region, is the first and only U.S. FDA-approved HIV-1 fusion inhibitor by competitively inhibiting CHR/NHR interaction and thereby preventing the formation of 6-HB.\textsuperscript{15,16} Another CHR-derived fusion inhibitor, MTSC22EK\textsuperscript{17} (Figure 28F and 28I), adds an MT hook to the CHR-derived peptide and was reported to exhibit activity even more potent than that of T20.

However, regular peptides, including T20, are rapidly degraded \textit{in vivo} and must be injected twice daily in large doses.\textsuperscript{18,19} Moreover, T20-resistant viruses are rapidly emerging and further limiting its application.\textsuperscript{20} Several studies have focused on improving stability and
efficiency of T20 by introducing a mutation to T20,21 adding an N-terminal anchor,22 C-terminal tails,23,24 or both22 and using staple strategy;25 however, these strategies still fail to eliminate the inherent drawbacks of natural peptides. In contrast, foldamers based on unnatural frameworks have attracted significant attention as alternative strategies to design novel protein-protein inhibitors (PPIs) to mimic the 3D structure and function of the natural peptide, but with much-enhanced stability and sequence diversity,26-35 including those inhibiting HIV-1 fusion.36,37 Nonetheless, foldamers bearing regular amino acid residues still suffer from low enzymatic stability,36 whereas those based on completely unnatural frameworks exhibited deteriorated activity (µMs) resulting from the difference in folding parameters between foldamers and the canonical α-helix backbone.37

To address challenges encountered in developing inhibitors to modulate PPIs, our group recently developed a class of foldamer termed γ-AApeptides (oligomers of N-acylated-N-aminoethyl amino acids).38 To date, γ-AApeptides have demonstrated remarkable resistance to proteolytic degradation, as well as enormous chemical diversity,39 affording their suitability as candidates for various biological functions.5,40-43 As a subclass of γ-AApeptides, sulfonyl-γ-AApeptides (Figure 28A) not only retain the aforementioned advantages, but also adopt well-defined helical structures.44-47 For instance, homogeneous L-sulfonyl-γ-AApeptides fold into left-handed 4_{14} helix with a helical pitch of 5.1 Å (Figure 28B to 28E), which is similar to that of α-helix (5.4 Å). This robust helicity is stabilized by both intramolecular hydrogen bonding and intrinsic turn-forming sulfonamido moieties.44,47 More importantly, four side chains are found in each helical turn, also similar to α-helix at 3.6 side chains/turn. Indeed, the side chains align perfectly with the four faces of sulfonyl-γ-AApeptide helical scaffold (Figure 28D). As such, sulfonyl-γ-AApeptides could present a unique molecular scaffold capable of projecting functional groups to mimic critical side chains on multiple faces of the α-helix.48-52
Figure 27 The fusion process of HIV-1 infection and the proposed inhibitory mechanism of sulfonyl-γ-AApeptides

(A) Schematic view of the HIV-1 gp41 molecule. FP, fusion peptide; NHR, N-terminal heptad repeat; PFD, pocket-forming domain; CHR, C-terminal heptad repeat; MPER, membrane-proximal external region; TM, transmembrane domain; and CP, cytoplasmic domain. (B) Model of HIV-1 gp41-mediated membrane fusion. (C) Side view of crystal structure of six-helix bundles formed by NHR in complex with CHR (PDB code 3F4Y). (D) Binding interaction of key residues on CHR (green) with NHR (magenta). (E) Proposed mechanism of sulfonyl-γ-AApeptides to inhibit the infection of HIV-1 virus.
Nonetheless, the ability of sulfonyl-γ-AApeptides to disrupt intramolecular PPIs remains elusive, even though such PPIs are involved in a wide variety of virus fusion processes.\(^8,53,54\) Therefore, we decided to explore the design principle of sulfonyl-γ-AApeptide for protein surface recognition by using HIV-1 gp41 fusion as the model system. Accordingly, we employed homogeneous sulfonyl-γ-AApeptides to mimic the binding mode of MTSC22EK (Figure 28I).\(^17\) We had previously reported a modified MTSC22EK sequence bearing backbone hydrocarbon stapling and a sulfonyl-γ-AA residue. Although the sequence exhibited anti-HIV-1 activity comparable to that of MTSC22EK, the sequence proved to be susceptible to enzymatic hydrolysis owing to its canonical peptide-based molecular scaffold, resulting in short half-life (t\(1/2 < 20\) min) (Figure 28L).\(^55\) Herein, we report the design of HIV-fusion inhibitors based on homogeneous sulfonyl-γ-AApeptides on a completely unnatural framework to disrupt 6-HB formation and to inhibit HIV-1 fusion with high potency and remarkable stability.

4.2 Results

4.2.1 Structure of NHR/CHR in 6-HB of gp41

As shown in Figure 27C, during the HIV-1 fusion process, the NHR domain forms an interior, parallel coiled-coil trimer, while the CHR domain packs in an oblique, antiparallel manner into highly conserved, hydrophobic grooves on the surface of this trimer. Both a deep pocket and neighboring sub-pocket were identified at the C-terminal region of the NHR trimer. Both were determined to be essential for stability of the 6-HB structure, and both potently interacted with the WWI motif in the pocket binding domain (PBD) of CHR domain (Figure 27D). MTSC22EK, which is derived from the CHR domain, exhibited potent inhibitory activity against diverse HIV-1 strains and a higher genetic barrier to resistance.\(^56\) Met-626 and Thr-627 in MTSC22EK adopt a
unique hook-like structure to stabilize the interaction between inhibitor and the deep pocket on the NHR (Figure 28I). It was thought that Met-626 could accommodate the hydrophobic groove between NHR and CHR domain and that Thr-627 could form the hydrogen bond between its hydroxyl side chain and the backbone NH group of the downstream residue Glu to stabilize the interaction.

Figure 28 Design of sulfonyl-γ-AApeptides mimicking MTCSC22EK

(A) Structure of sulfonyl-γ-AApeptides. (B) The crystal structure of a sulfonyl-γ-AApeptide. (C) Top view of (B). (D and E) Schematic representation of the distribution of side chains from sulfonyl-γ-AApeptides. (D) Top view. (E) Side view, helical wheel. (F) Structure of MTSC22EK (white). (G) sulfonyl-γ-AApeptide mimic 12 (green). (H) Overlay of key binding residues between (F) and (G). (I) Binding interaction of key residues of MTSC22EK (white) with NHR (PDB code: 3VU6). (J) Binding interaction of key residues of sulfonyl-γ-AApeptide mimic 12 (green) with NHR. (K) Superimposition of 12 (green) with critical residues of MTSC22EK (white) on the binding surface of NHR. (L) Comparison of MTSC22EK with our previous work and this work.
4.2.2 **Design of sulfonyl-γ-AApeptides**

MTSC22EK was therefore chosen as our prototype peptide for the design of sulfonyl-γ-AApeptides. MTSC22EK interacts with the NHR using its hydrophobic residues (626M, 627T, 628W, 631W, 635I, 639T, 642I and 645I), whereas E and K residues mainly enhance the solubility and stability of the helical structure through potential charge-charge attraction (Figure 28I). Thus, we introduced 6 chiral hydrophobic residues at the 2a, 4a, 6a, 8a, 10a and 12a positions (Figure 28D, Figure 28E and Figure 28G), all at the same face of the helix, to mimic those hydrophobic residues in MTSC22EK (Figure 28H), which, in turn, were expected to bind with the deep hydrophobic pocket of NHR (Figure 28J and Figure 28K). Then, similar to E and K residues in MTSC22EK, we introduced alternating negative charges and positive charges at the other two faces to form a salt bridge to stabilize our sequences, as well as increase solubility (Table 5).

We initially designed sulfonyl-γ-AApeptide 2 and tested it with the HIV-1 authentic infection assay. In this assay, the IC\textsubscript{50} of 2 was determined to be 30 µM, which is three orders of magnitude lower than that of T20 (30 µM vs. 0.03 µM). Even though the activity is very moderate, the results suggested that using sulfonyl-γ-AApeptides to inhibit HIV-1 entry is a viable strategy. Next, we asked whether the Trp, Trp and Ile side chains (2a, 4a, 8a) were essential to the inhibitory activity of sulfonyl-γ-AApeptide against the virus or not. To address this question, we removed these three groups in the sequence and found that sequence 3 completely lost its activity, suggesting that these side chains in CHR are crucial to the design of our molecular scaffold. We next set out to explore the effect of side chains at different positions. First, studies were carried out to probe the effect of negative charge at positions 3b, 7b and 11b based on sequence 2. We designed and synthesized sequences 4, 5, 6, 7 in which these three negative charges were replaced with the Ala side chain individually and collectively. All four sequences lost activity, indicating that these
three negative charges may play a critical role in stabilizing structure and activity. To investigate the importance of the middle chiral side chain Tyr, as well as the side chain at position 8b, we replaced the hydrophobic side chain of Tyr with the side chain of Ser (sequence 8), which is polar, but uncharged, and this led to completely loss of activity. The activity also disappeared when replacing the bulky side chain at position 8b with a methyl group (sequence 9). It is well known that the N-terminal residues Met-626 and Thr-627 of MTSC22EK adopt a unique hook-like structure (M-T hook), strengthening the stability and antiviral activity of MTSC22EK.\textsuperscript{17} This was also true of sequence 10; however, when the first building block (mimicking MT) was removed from 2, sequence 10 completely lost its activity. Consequently, we screened different side chains at position 1b to identify the optimal group able to mimic the function of MT in MTSC22EK. While a small group at 1b (sequence 11) moderately decreased activity, replacement with a bulkier 4-methoxybenzene group (sequence 12) led to a ~4-fold increase in anti-HIV-1 activity compared to 2. Interestingly, when two more building blocks were appended at the C-terminal of sequence 12, the new construct, sequence 13, exhibited deteriorated activity.

Lipid conjugation is a promising strategy to increase the activity of HIV-1 fusion inhibitors by anchoring the lipopeptides to the target cell membrane to raise the local concentration of inhibitors at the viral entry site.\textsuperscript{22,57} To this end, sequences 2 and 12 were modified with C18 hydrocarbon tails at the C-terminus, leading to 2’ and 12’ that exhibited significantly enhanced activity with IC\textsubscript{50} values of 0.98 µM and 0.18 µM, respectively. To the best of our knowledge, 2’ and 12’ are among the most potent oligomeric foldamers based on completely unnatural molecular frameworks. To ensure that the sequences are specific toward HIV-1, instead of mammalian cells, we assessed the cytotoxicity of these sequences toward the mammalian MT-2 cell line and found that the half cytotoxic concentration (CC\textsubscript{50}) of most sequences was much higher than their anti-
fusion activity. The selectivity index (SI=CC50/IC50) of the two most potent sequences 2’ and 12’ is > 40- and 106-fold, respectively, suggesting that sequences 2’ and 12’ are promising HIV-1 fusion inhibitors with negligible toxic effects.

4.2.3 Lipo-sulfonyl-γ-AApeptides exhibit potent inhibitory activity against HIV-clinical isolates

The continuous emergence of drug-resistant HIV-1 mutants is a major hurdle for current clinical anti-HIV-1 drugs. This calls for the development of new anti-HIV-1 therapeutic agents to circumvent such drug resistance. Therefore, apart from laboratory-adapted HIV-1 IIIB strains, we also tested the inhibitory activity of 2’ and 12’ against a panel of HIV-1 clinical isolates. We first investigated the inhibitory activity of sequences 2’ and 12’ against five pseudotyped HIV-1 clinical isolates. In these HIV-1 pseudovirus assays, both sequences showed potent inhibitory activity against all tested strains with IC50 values at low nanomolar ranging from 46.7 to 154.0 nM, similar to the inhibitory activity of T20 (Table 6). We also tested the antiviral potency of sequences 2’ and 12’ against another two clinical isolates of authentic virus. As shown in Table 7, both sequences displayed highly potent activities against diverse HIV-1 clinical isolates with IC50 at the nanomolar ranging from 81.3 to 940.2 nM. These results suggested that sequences 2’ and 12’ have similar potency against HIV-1 clinical isolates, suggesting that foldamers on the unnatural sulfonyl-γ-AApeptide backbone could be potent anti-HIV-1 agents and successfully overcome drug resistance. Taken together, these results show that major HIV-clinical isolates could be efficiently blocked by these two lead sequences, further indicating that sequences 2’ and 12’ could be promising next-generation broad-spectrum anti-HIV-1 reagents.
Table 5 Anti-HIV-1 activities (IC\textsubscript{50}) for inhibiting HIV-1 IIIB strains replication and half-maximal cytotoxicity concentration (CC\textsubscript{50}) of sulfonyl-\textgamma -Apeptides

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
<th>IC\textsubscript{50} (\textmu M)</th>
<th>CC\textsubscript{50} (\textmu M)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DDDDDDDD</td>
<td>0.001</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DDDDDDDD</td>
<td>30±7</td>
<td>&gt;40</td>
<td>&gt;1.3</td>
</tr>
<tr>
<td>3</td>
<td>DDDDDDDD</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>DDDDDDDD</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td></td>
</tr>
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<td>5</td>
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<td>&gt;100</td>
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<td>9</td>
<td>DDDDDDDD</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
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<td>10</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>DDDDDDDD</td>
<td>38.93±8.0</td>
<td>&gt;100</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>12</td>
<td>DDDDDDDD</td>
<td>4</td>
<td>&gt;40</td>
<td>&gt;10</td>
</tr>
<tr>
<td>13</td>
<td>DDDDDDDD</td>
<td>15.72±1.3</td>
<td>&gt;100</td>
<td>&gt;6.3</td>
</tr>
<tr>
<td>2'</td>
<td>DDDDDDDD</td>
<td>0.98±0.16</td>
<td>&gt;40</td>
<td>&gt;41</td>
</tr>
<tr>
<td>12'</td>
<td>DDDDDDDD</td>
<td>0.18±0.10</td>
<td>19.30±1.89</td>
<td>106</td>
</tr>
</tbody>
</table>

Note: The IC\textsubscript{50} was assessed by the inhibitory activity against \textit{in vitro} infection by authentic HIV-1 virus. SI=IC\textsubscript{50}/CC\textsubscript{50}. 

118
Table 6 Lipo-sulfonyl-γ-AApeptides’ inhibitory activity against HIV-1 clinical isolate
pseudovirus infection (IC₅₀)

<table>
<thead>
<tr>
<th>Name</th>
<th>Primary Env</th>
<th>Subtype</th>
<th>Inhibition IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2’</td>
</tr>
<tr>
<td>1</td>
<td>AG</td>
<td>CRF02_AG Clone 278</td>
<td>89.0±11.2</td>
</tr>
<tr>
<td>2</td>
<td>D</td>
<td>QD435.100M.ENV.E1</td>
<td>84.9±17.1</td>
</tr>
<tr>
<td>3</td>
<td>AG</td>
<td>CRF02_AG Clone 33</td>
<td>123.8±8.7</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>Q259env.w6</td>
<td>154.0±14.1</td>
</tr>
<tr>
<td>5</td>
<td>D</td>
<td>QA013.701.ENV.M12</td>
<td>46.7±8.6</td>
</tr>
</tbody>
</table>

Table 7 Lipo-sulfonyl-γ-AApeptide inhibitory activity against HIV-1 clinical isolate
authentic infection (IC₅₀)

<table>
<thead>
<tr>
<th>Clade</th>
<th>Isolate laboratory ID</th>
<th>Co-receptor</th>
<th>Inhibition IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2’</td>
</tr>
<tr>
<td>A</td>
<td>MN/H9 (84US_MNp)</td>
<td>X4</td>
<td>81.3±13.5</td>
</tr>
<tr>
<td>B</td>
<td>US4/GS 007 (91US_4)</td>
<td>R5</td>
<td>87.8±10.2</td>
</tr>
</tbody>
</table>

4.2.4 Sulfonyl-γ-AApeptides form stable helical structures

It was thought that the inhibitory activity of sulfonyl-γ-AApeptides stems from their 3D-structure. As confirmation, CD spectra were obtained for those sequences (100 µM) in PBS buffer. As shown in Figure 29A, most sequences revealed a positive maximum at around 208 nm, which is a characteristic cotton effect of a well-defined left-handed 4₁⁴ helix.

4.2.5 Sequences 2’ and 12’ disrupt NHR/CHR interaction

To gain insight into the molecular mechanism underlying the antiviral activity of sulfonyl-γ-AApeptides, CD spectra were carried out to analyze the secondary structure of the complexes formed between sulfonyl-γ-AApeptides and N36 target. N36, which is an NHR-derived peptide
that mimics the NHR region of gp41, interacts with the CHR-derived peptide C34 to form 6-HB. As shown in Figure 29B, neither C34 nor N36 alone adopted defined helical structures in solution owing to the absence of the signatures of 208 and 222 nm. However, complexation of C34 and N36 led to significantly more pronounced minimums at 208 and 222 nm, suggesting formation of the helix bundle. Similarly, the spectra of the mixtures 12’/N36 (Figure 29C) exhibited a significant conformational change of the secondary structure of N36, indicating that sulfonaryl-γ-AApeptides strongly interact with the NHR-derived peptide N36. In particular, the mixture of sulfonaryl-γ-AApeptides and N36 revealed similar negative maximums at 208 and 222 nm (Figure 29C) in the presence of 12’ in comparison to C34/N36, suggesting that 12’ is analogous to C34 and that it significantly enhanced the helicity of N36 peptide. Overall, our CD studies strongly supported the idea that sulfonaryl-γ-AApeptides could mimic the function of MTSC22EK to inhibit fusion of the HIV-1 virus. To further confirm the binding between sulfonaryl-γ-AApeptides and N36, a fluorescence polarization assay was conducted. As shown in Figure 29D, the Kd of 12’ and N36 was determined to be 0.67 μM, demonstrating excellent binding affinity with N36. After the addition of 5 μM C34, the binding affinity reduced to 46.0541 μM (Figure 29E). These results demonstrated that 12’ binds to N36 in competition with C34, suggesting that these helical sulfonaryl-γ-AApeptides could mimic the binding mode of C34.

4.2.6 Parallel artificial membrane permeability assays (PAMPA) of sequences 2’ and 12’

To determine whether sequences 2’ and 12’ could be used as potential anti-HIV-1 drug candidates or not, we applied the parallel artificial membrane permeability assay to evaluate them. The PAMPA assay has been approved for the assessment of transmembrane, non-energy dependent, diffusion of drugs, making it a reasonable assay for the prediction of passive drug
absorption *in vivo*.\(^{58}\) As such, we conducted the gastrointestinal tract (GIT) parallel artificial membrane permeability assay (PAMPA-GIT) to evaluate the oral route of sequences \(2'\) and \(12'\) based on gastrointestinal absorption rate. Then we conducted the blood-brain barrier (BBB) parallel artificial membrane permeability assay (PAMPA-BBB) to determine the ability of sequences \(2'\) and \(12'\) to penetrate the BBB.

![Figure 29 Mechanistic studies of the inhibitory activity of sulfonyl-γ-AApeptides](image)

- (A) CD spectra of sulfonyl-γ-AApeptides 2-12' measured at 100 μM, room temperature in PBS buffer. (B and C) Biophysical characterization of peptides, sulfonyl-γ-AApeptides and their derivatives by CD spectroscopy, (B) N36, C34 and N36/C34. (C) \(12'\), N36, and \(12'\)N36. (D) Binding affinity of \(12'\) to N36, as measured by fluorescence polarization (FP) assay. (E) Binding affinity of \(12'\) to N36 (adding 5 μM C34), as measured by fluorescence polarization (FP) assay.

For the PAMPA-GIT assay, we tested our sequences in different pH conditions because human intraluminal pH is different in stomach, duodenum, ileum, cecum, and rectum. We used carbamazepine, a fully bioavailable drug, as positive control. It exhibited favorable permeability at pH 5.0, 6.2 and 7.4 for \(P_{\text{app}}\) values with \(134.41 \times 10^{-6} \text{ cm/s}, 141.28 \times 10^{-6} \text{ cm/s}, \) and \(152.27 \times 10^{-6} \text{ cm/s}\).
6 cm/s, respectively, even at concentration as low as 50 µM (Figure 30A). Antipyrine served as a negative control by its poor bioavailability. As expected, it showed very low permeability (Papp < 1 × 10⁻⁶ cm/s) at different pH conditions, even at very high concentrations (200 µM) (Figure 30A).

As shown in Figure 30A, sequence 2’ (50 µM) displayed favorable permeability without pH-dependence for Papp values at 601.58 × 10⁻⁶ cm/s, 616.75 × 10⁻⁶ cm/s and 390.33 × 10⁻⁶ cm/s at pH 5.0, 6.2 and 7.4, respectively. Sequence 12’ (50 µM) also showed favorable permeability without pH-dependence for Papp values at 609.92 × 10⁻⁶ cm/s, 522.33 × 10⁻⁶ cm/s and 520.47 × 10⁻⁶ cm/s at pH 5.0, 6.2 and 7.4, respectively. These data suggested that sequences 2’ and 12’ have oral bioavailability potential for future drug development.

The brain is thought to be a sanctuary site of HIV-1 virus, and most anti-HIV-1 drugs cannot penetrate the BBB, thus contributing to the therapeutic failure of many anti-HIV-1 drugs.59 As such, we evaluated the ability of our sequences to penetrate the BBB by PAMPA-BBB assay. As shown in Figure 30B, verapamil, our positive control, is a drug that easily penetrates the BBB with a Papp value of 148.88 × 10⁻⁶ cm/s at a concentration of 50 µM (Papp value > 20 × 10⁻⁶ cm/s is considered favorable permeability). However, Theophylline, our negative control, hardly passes through the BBB, showing a low permeability with a Papp value < 10 × 10⁻⁶ cm/s, even at high concentration (250 µM). In comparison, sequences 2’ (25 µM) and 12’ (15 µM) both exhibited favorable permeability with Papp values with 234.40 × 10⁻⁶ cm/s and 256.20 × 10⁻⁶ cm/s, respectively (Figure 30B). Since sequences 2’ and 12’ could easily penetrate BBB, both are promising next-generation anti-HIV-1 drugs to treat and control of HIV-1 within the central nervous system (CNS).
4.2.7 **Sequences 2' and 12' are much more resistant than MTSC22EK to enzymatic degradation**

T20 and MTSC22, as well as other fusion inhibitors, have shown remarkable susceptibility to degradation by proteases. Therefore, the resistance of sulfonyl-γ-AApeptides to proteolytic degradation was evaluated. As sequences 2' and 12' displayed very low fluorescence absorption at 215 and 254 nm, we used sequences 2 and 12 to represent their stability as they had identical structures, except for the carbon tails. As shown in Figure 31, MTSC22EK was completely degraded after incubating with 0.1 mg/mL pronase in 100 mM ammonium bicarbonate buffer at 37 °C for 24 h. By contrast, sequences 2 and 12 showed no detectable degradation after incubation for 24 h. These results demonstrated that sulfonyl-γ-AApeptide is against enzymatic degradation,
qualifying it as a potential therapeutic agent in highly active antiretroviral therapy (HAART). In our next studies, we will test these two lead sequences for their activities in vivo.

4.3 Discussion

Despite the efficacy of HAART therapy, HIV-1 remains one of the biggest threats to human health. HIV-1 drug resistance is a significant contributor to the clinical failure of HAART therapy. It is vitally necessary to find new drugs that can eradicate the virus with little drug resistance and, thus, increase the efficacy of the HAART therapy. Biochemists have recently made great strides in the successful use of peptidomimetics, such as β-peptide, aromatic oligoamides and others, to produce innovative medications that inhibit HIV-1 in vitro and, thus, enhance drug resistance. Peptidomimetics with heterogeneous backbones, however, had limited enzymatic stability, while those based on homogeneous unnatural backbones always showed modest activity.

In this study, we used sulfonyl-γ-AA peptides-based helical foldamers to mimic a bioactive peptide (MTSC22EK) to disrupt the formation of 6-HB and thus block HIV-1 infection. Recently, we have successfully used sulfonyl-γ-AA peptides as novel helical mimetics to modulate many significant intracellular and extracellular protein-protein interactions. The current design was based on the interaction between MTSC22EK and NHR. Since the hydrophobic residues of Met626, Thr627, Trp628, Trp628, Ile635, Thr639, Ile642 and Ile646 are critically involved in the binding interaction of MTSC22EK with NHR, we maintained these residues in our sulfonyl-γ-AA peptides mimetics. We also introduced alternating hydrophilic groups in the other three faces of our helical foldamers to enhance the stability and solubility. Finally, we successfully mimicked the MT hook as a building block to significantly improve the anti-HIV-1 activities of our designed sequences, including 2, 11, 12 and 13, all of which demonstrated good activity in vitro to inhibit
HIV-1 fusion. Especially, after modification with C18 carbon tail, sequences 2' and 12' demonstrated much more potent activity than the original sequences (2 and 12) by > 30- and 20-fold, respectively, with excellent SI (> 40- and 106-fold, respectively). Moreover, sequences 2' and 12' exhibited potent activity against most HIV-1 clinical isolates. Mechanistic studies suggested that sequences 2' and 12' tightly bind with NHR to stop the formation of 6-HB and thus inhibit HIV-1 fusion, just like T20 (Figure 27E). Furthermore, sequences 2' and 12' displayed favorable permeability based on both PAMPA-BBB and PAMPA-GIT assays and exhibited much more resistance to proteolytic enzymes. Collectively, these results suggested that sequences 2' and 12' are promising next-generation candidates for in vivo studies. These findings further demonstrated the potential ability of sulfonyl-γ-AA peptides to mimic the bioactive peptide for the development of antiviral regents.

4.4 Conclusion

In summary, we have constructed a series of unprecedented left-handed helical sulfonyl-γ-AA peptides to disrupt the formation of 6-helix bundle structure and, thus block HIV-1 virus entry to the target cells. Extensive data from efficacy evaluation and mechanistic studies strongly suggested that these sulfonyl-γ-AA peptides could mimic helical structures of CHR and therefore disrupt CHR/NHR intramolecular interaction in HIV. Overall, sulfonyl-γ-AA peptides could be the potential candidates for anti-HIV-1 therapeutic agents for the future study, as evidenced by their broad-spectrum anti-HIV-1 activity to many HIV-clinical isolates and proteolytic stability. Moreover, the best two lipo-sulfonyl-γ-AA peptides display favorable PAMPA permeability, as well as promising oral bioavailability and therefore could potentially treat and control HIV-1 within the central nervous system (CNS). The strategy of sulfonyl-γ-AA peptides as an antiviral
scaffold to inhibit viral fusion could be extended to tackle a variety of viral targets, as well as other variety PPIs.

Figure 31 Stability studies of MTSC22EK and sulfonyl-γ-AApeptide. HPLC traces of indicated control sequences and sequences incubated in pronase for 24 h
4.5 Materials and methods

4.5.1 Synthesis of sulfonyle-γ-AApeptide building blocks

4.5.1.1 General information

Fmoc-protected amino acids were purchased from Chem-impex (Wood Dale, IL). All other chemicals and solvents were purchased and directly used from Fisher Scientific, Sigma-Aldrich, or Oakwood. Thin-layer chromatography was performed on Sorbtech TLC plates (silica gel w/UV254) and visualized with UV-light 254 nm. Flash column chromatography was performed with ICN silica gel (60 Å, 230-400 mesh, 32-63 um). $^1$H NMR spectra were recorded at 600 MHz using the internal standard. $^{13}$C NMR spectra were recorded at 150 MHz using TMS as the internal standard. The mass of each building block was determined by high-resolution mass spectrometry detected by High-resolution MS (HRMS) on Agilent 6540 LC/QTOF.

4.5.1.2 Synthesis

Building blocks 1-12 were synthesized based on the previous report routes.$^{48-51}$ Building block 13 was synthesized based on Figure 32.
4.5.2 Characterization of sulfonyle-γ-4Apeptide building blocks

(S)-N-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(methylthio)butyl)-N-(4-methoxyphenyl)sulfonyl)glycine (BB10). $^1$H NMR (600 MHz, DMSO-d6): δ 12.72 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.74 – 7.69 (m, 2H), 7.67 (dd, J = 7.6, 2.8 Hz, 2H), 7.41 (td, J = 7.5, 3.4 Hz, 2H), 7.37 – 7.28 (m, 2H), 7.19 (d, J = 8.7 Hz, 1H), 7.13 – 7.04 (m, 2H), 4.31 (dd, J = 10.4, 7.0 Hz, 1H), 4.28 – 4.18 (m, 2H), 3.81 (s, 2H), 3.68 (tq, J = 10.7, 7.3, 5.2 Hz, 1H), 3.34 (s, 2H), 3.26 (dd, J = 14.2, 6.9 Hz, 1H), 3.09 (dd, J = 14.1, 7.0 Hz, 1H), 2.45 – 2.37 (m, 1H), 2.33 (ddd, J = 14.2, 9.7 Hz, 1H), 2.00 (d, J = 12.8 Hz, 3H), 1.81 (dt, J = 13.5, 6.2, 3.6 Hz, 1H), 1.54 (dtd, J = 14.2, 9.7, 4.9 Hz, 1H). $^{13}$C NMR (150 MHz, DMSO-d6): δ 170.58, 162.91, 156.28, 144.36, 144.26, 141.21, 131.47, 130.04, 129.67, 128.08, 127.48, 125.63, 120.57, 114.75, 65.64, 56.08, 52.01, 49.36, 49.00, 47.26, 31.74, 30.28, 15.12. HRMS (ESI) ([M+H]$^+$) Calcd. for C$_{29}$H$_{32}$N$_2$O$_7$S$_2$: 585.1651, found 585.1665.

(S)-N-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(methylthio)butyl)-N-(methylsulfonyl)glycine (BB11). $^1$H NMR (600 MHz, DMSO-d6): δ 12.88 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.69 (t, J = 6.4 Hz, 2H), 7.42 (t, J = 7.4 Hz, 2H), 7.33 (tdd, J = 7.5, 3.4, 1.2 Hz, 2H), 7.23 (d, J = 8.9 Hz, 1H), 4.35 (t, J = 6.7 Hz, 2H), 4.22 (t, J = 6.8 Hz, 1H), 3.98 (s, 2H), 3.73 (dt, J = 9.2, 4.4 Hz, 1H), 3.28 (dd, J = 14.4, 5.8 Hz, 1H), 3.15 (dd, J = 14.4, 8.2 Hz, 1H), 2.94 (s, 3H), 2.47 – 2.33 (m, 2H), 2.03 (s, 3H), 1.76 (dt, J = 9.8, 6.3, 2.8 Hz, 1H), 1.56 (dtd, J = 14.2, 9.5, 5.0 Hz, 1H). $^{13}$C NMR (150 MHz, DMSO-d6): δ 171.24, 156.43, 144.37, 141.23, 128.08, 127.50, 125.61, 120.56, 65.63, 60.23, 51.36, 49.30, 48.78, 47.29, 31.87, 30.26, 15.14. HRMS (ESI) ([M+H]$^+$) Calcd. for C$_{23}$H$_{28}$N$_2$O$_7$S$_2$: 493.1389, found 493.1377.

(S)-N-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(methylthio)butyl)-N-(isobutylsulfonyl)glycine (BB12). $^1$H NMR (600 MHz, DMSO-d6) δ 12.85 (s, 1H), 7.90 (d, J = 7.6 Hz, 2H),
7.69 (t, J = 7.0 Hz, 2H), 7.42 (t, J = 7.4 Hz, 2H), 7.33 (tdd, J = 7.4, 3.2, 1.1 Hz, 2H), 7.24 (d, J = 8.9 Hz, 1H), 4.36 (dd, J = 10.5, 7.0 Hz, 1H), 4.29 (dd, J = 10.6, 6.9 Hz, 1H), 4.22 (t, J = 6.9 Hz, 1H), 3.98 (d, J = 3.2 Hz, 2H), 3.72 (dt, J = 9.5, 4.6 Hz, 1H), 3.30 (dd, J = 14.4, 5.9 Hz, 1H), 3.17 (dd, J = 14.4, 8.2 Hz, 1H), 3.04 – 2.93 (m, 2H), 2.47 – 2.33 (m, 2H), 2.13 – 2.04 (m, J = 6.7 Hz, 1H), 2.03 (s, 3H), 1.77 (dddd, J = 13.4, 10.0, 6.6, 3.7 Hz, 1H), 1.56 (dtd, J = 14.3, 9.5, 5.0 Hz, 1H), 0.98 (t, J = 6.2 Hz, 6H). 13C NMR (150 MHz, DMSO-d6): δ 171.26, 156.43, 144.37, 141.22, 128.09, 127.51, 125.64, 120.57, 65.68, 59.35, 51.31, 49.23, 48.52, 47.29, 31.90, 30.27, 24.69, 22.66, 15.15. HRMS (ESI) ([M+H]⁺) Calcd. for C26H34N2O6S2: 535.1858, found 535.1856.

(S)-N-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(tert-butoxy)-5-oxopentyl)-N-((4-(((4-((1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)amino)benzyl)oxy)carbonyl)phenyl)sulfonyl)glycine (BB13). 1H NMR (600 MHz, DMSO-d6): δ 15.28 (s, 1H), 8.13 (d, J = 8.5 Hz, 2H), 7.93 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 7.5 Hz, 2H), 7.65 (t, J = 6.7 Hz, 2H), 7.55 (d, J = 8.1 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.36 – 7.28 (m, 4H), 7.12 (d, J = 8.9 Hz, 1H), 5.40 (s, 2H), 4.30 – 4.23 (m, 1H), 4.18 (h, J = 7.0 Hz, 2H), 4.06 (d, J = 12.3 Hz, 2H), 3.67 – 3.61 (m, 1H), 3.33 (dd, J = 14.3, 5.9 Hz, 1H), 3.20 (dd, J = 14.3, 8.1 Hz, 1H), 2.40 (s, 4H), 2.15 – 2.02 (m, 2H), 1.71 (dt, J = 10.0, 6.7 Hz, 3H), 1.46 (ddt, J = 14.5, 9.2, 5.5 Hz, 2H), 1.37 (s, 9H), 1.01 (s, 6H), 0.69 (d, J = 6.6 Hz, 6H). 13C NMR (150 MHz, DMSO-d6): δ 175.46, 172.26, 170.22, 165.00, 158.88, 158.64, 156.24, 144.32, 144.23, 144.16, 141.18, 136.83, 135.65, 133.29, 130.52, 129.44, 128.06, 127.84, 127.47, 126.93, 125.58, 120.56, 107.60, 80.03, 66.51, 65.65, 52.80, 51.75, 49.18, 48.60, 47.22, 37.83, 31.77, 30.11, 29.28, 28.17, 27.47, 22.78. HRMS (ESI) ([M+H]⁺) Calcd. for C53H61N3O12S: 964.3976, found 964.3978.
4.5.3 *Sulfono-γ-AApeptides preparation*

4.5.3.1 General information

Solid-phase synthesis was conducted in peptide synthesis vessels on a Burrell Wrist-Action shaker. γ-AApeptides were analyzed and purified on a Waters Breeze 2 HPLC system installed with both the analytic module (1 mL/min) and preparative module (16 mL/min) by employing a method using a 5-100% linear gradient of solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in water) over 40 min, followed by 100% solvent B over 10 min. Then the pure peak was collected and lyophilized on a Labcono lyophilizer. The purity of the sequences was determined to be > 95% by analytical HPLC. High-resolution mass spectra were obtained on a Bruker UltraFleXtreme MALDI-TOF/TOF.

![Diagram of sulfono-γ-AApeptide building blocks BB1-BB13](image_url)

*Figure 33 Sulfono-γ-AApeptide building blocks BB1-BB13*
4.5.3.2 Synthesis

Sulfonyl-γ-AApeptide synthesis was carried out on 100 mg of Rink Amide-MBHA resin (0.646 mmol/g) under room temperature at atmospheric pressure.\textsuperscript{48-51} Resin was swelled in dimethylformamide (DMF) for 5 min before use, followed by treatment with 20% piperidine/DMF solution (2 ml) to remove the Fmoc-protecting group and washed afterward three times with dichloromethane (DCM) and three times with DMF. A premixed solution of sulfonyl-γ-AApeptide building block (2 equiv), HOBt (4 equiv), and DIC (4 equiv) in 2 mL of DMF was added to the resin and shaken for 4 hours to complete the coupling reaction. After washing with DCM and DMF, the resin was treated with 20% piperidine/DMF solution for 15 min (× 2). Another sulfonyl-γ-AApeptide building block (2 equiv) was attached on the resin following the procedure in the first coupling step, and the Fmoc protecting group was removed after the coupling reaction was done. The reaction cycles were repeated until the desired sulfonyl-γ-AApeptides were synthesized. The N-terminus of the sequence was capped with acetic anhydride (1 mL) in pyridine (2 mL) (15 min × 2). After that, a solution of hydrazine monohydrate in DMF (2% v/v, 3 mL) was added to peptide synthesis vessels, shaken for 3 min, followed by repeating the procedure 4 times to completely deprotect the Dmab protecting group. For the sulfonyl-γ-AApeptide with C18 tail, Fmoc-Lys(Dde)-OH was first attached to the Rink amide resin, followed by treatment with NH\textsubscript{2}OHꞏHCl and imidazole in NMP solution for 1 hour, followed by washing three times with dichloromethane (DCM) and three times with DMF. Then stearic acid, HOBt and DIC were added to react with side chain of Lys. The rest of the procedure was exactly the same as that performed for regular sulfonyl-γ-AApeptide synthesis. Finally, sulfonyl-γ-AApeptide was cleaved by 82.5% TFA, 5% phenol, 5% water, 5% thioanisole and 2.5% 1,2-ethanedithiol for 3 h. The cleavage solution was collected, and the beads washed with DCM (3 mL × 2). The solution was combined and evaporated under
air flow to give the crude product, which was analyzed and purified by a Waters HPLC system at 1 and 16 mL/min flow rates for analytic and preparative HPLC, respectively. The gradient eluting method of 5-100% of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 50 min was performed. The pure peptides were then collected and lyophilized on a Labconco lyophilizer; the purity of the sequences was determined to be > 95% by analytical HPLC.

Figure 34 Synthetic routes of sulfono-γ-AApeptides and lipid- sulfono-γ-AApeptides
4.5.3.3 Characterization of sulfonyl-\(\gamma\)-AApeptides

1

Chemical Formula: \(\text{C}_{143}\text{H}_{225}\text{N}_{33}\text{O}_{43}\text{S}\)

Theoretical Mol. Wt.: 3126.6210

Observed (MALDI-TOF): 3127.7068 (M+H\(^+\))

2

Chemical Formula: \(\text{C}_{160}\text{H}_{259}\text{N}_{31}\text{O}_{50}\text{S}_{13}\)

Theoretical Mol. Wt.: 3833.7790

Observed (MALDI-TOF): 3834.7209 (M+H\(^+\))
Chemical Formula: C$_{140}$H$_{241}$N$_{33}$O$_{53}$S$_{12}$

Theoretical Mol. Wt.: 3619.3660

Observed (MALDI-TOF): 3620.4325

Chemical Formula: C$_{154}$H$_{257}$N$_{31}$O$_{48}$S$_{13}$

Theoretical Mol. Wt.: 3727.6990

Observed (MALDI-TOF): 3728.9575 (M+H$^+$)
5

Chemical Formula: $C_{154}H_{257}N_{31}O_{48}S_{13}$

Theoretical Mol. Wt.: 3727.6990

Observed (MALDI-TOF): 3728.9922 (M+H$^+$)

6

Chemical Formula: $C_{154}H_{257}N_{31}O_{48}S_{13}$

Theoretical Mol. Wt.: 3727.6990

Observed (MALDI-TOF): 3728.7817 (M+H$^+$)
Chemical Formula: C\textsubscript{142}H\textsubscript{253}N\textsubscript{31}O\textsubscript{44}S\textsubscript{13}

Theoretical Mol. Wt.: 3515.5390

Observed (MALDI-TOF): 3516.3865 (M+H\textsuperscript{+})

Chemical Formula: C\textsubscript{154}H\textsubscript{255}N\textsubscript{31}O\textsubscript{50}S\textsubscript{13}

Theoretical Mol. Wt.: 3757.6810

Observed (MALDI-TOF): 3758.8630 (M+H\textsuperscript{+})
Chemical Formula: C\textsubscript{157}H\textsubscript{253}N\textsubscript{31}O\textsubscript{50}S\textsubscript{13}

Theoretical Mol. Wt.: 3791.6980

Observed (MALDI-TOF): 3792.7964 (M+H\textsuperscript{+})

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Chemical Formula: C\textsubscript{149}H\textsubscript{237}N\textsubscript{29}O\textsubscript{47}S\textsubscript{11}

Theoretical Mol. Wt.: 3539.3510

Observed (MALDI-TOF): 3540.7097 (M+H\textsuperscript{+})
Chemical Formula: C\textsubscript{157}H\textsubscript{253}N\textsubscript{31}O\textsubscript{50}S\textsubscript{13}

Theoretical Mol. Wt.: 3791.6980

Observed (MALDI-TOF): 3792.7064 (M+H\textsuperscript{+})

Chemical Formula: C\textsubscript{163}H\textsubscript{257}N\textsubscript{31}O\textsubscript{51}S\textsubscript{13}

Theoretical Mol. Wt.: 3883.7950

Observed (MALDI-TOF): 3884.8955 (M+H\textsuperscript{+})
Chemical Formula: C185H303N37O57S15
Theoretical Mol. Wt.: 4438.5610
Observed (MALDI-TOF): 4439.4874 (M+H+)

Chemical Formula: C184H305N33O52S13
Theoretical Mol. Wt.: 4228.4230
Observed (MALDI-TOF): 4229.1221 (M+H+)
**12'**

Chemical Formula: C$_{187}$H$_{303}$N$_{33}$O$_{53}$S$_{13}$

Theoretical Mol. Wt.: 4278.4390

Observed (MALDI-TOF): 4279.2974 (M+H$^+$)

---

**FITC-12'**

Chemical Formula: C$_{209}$H$_{319}$N$_{35}$O$_{58}$S$_{14}$

Theoretical Mol. Wt.: 4698.8780

Observed (MALDI-TOF): 4700.0386 (M+H$^+$)
4.5.3.4 HPLC purities and retention time of pure peptides

Table 8 HPLC purities and retention time of regular peptide and sulfonamido-γ-AApeptides\(^a\)

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Purity trace after HPLC purification (%)</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98.77</td>
<td>19.426</td>
</tr>
<tr>
<td>2</td>
<td>99.14</td>
<td>23.367</td>
</tr>
<tr>
<td>3</td>
<td>98.63</td>
<td>18.304</td>
</tr>
<tr>
<td>4</td>
<td>99.78</td>
<td>23.872</td>
</tr>
<tr>
<td>5</td>
<td>97.57</td>
<td>23.893</td>
</tr>
<tr>
<td>6</td>
<td>99.13</td>
<td>23.722</td>
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<td>7</td>
<td>98.47</td>
<td>22.760</td>
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<tr>
<td>8</td>
<td>99.29</td>
<td>23.478</td>
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<td>9</td>
<td>98.20</td>
<td>23.233</td>
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<td>22.388</td>
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<td>99.55</td>
<td>23.899</td>
</tr>
<tr>
<td>2'</td>
<td>99.83</td>
<td>32.994</td>
</tr>
<tr>
<td>12'</td>
<td>98.51</td>
<td>32.895</td>
</tr>
<tr>
<td>FITC-12'</td>
<td>98.46</td>
<td>34.287</td>
</tr>
</tbody>
</table>

\(^a\) The gradient eluting method of 5% to 95% of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 50 min was performed.

4.5.4 Circular dichroism spectroscopy

CD spectra were obtained on an Aviv 215 CD spectrometer using a 1 mm path length quartz cuvette.\(^5\) Lyophilized solid sequence was dissolved into PBS buffer and followed by dilution to give the desired concentration (100 μM) and solvent combination. Ten scans were averaged for each sample, independent experiments were conducted three times, and the spectra were averaged. The final spectra were normalized by subtracting the average blank spectra. Molar ellipticity \([\theta]\) (deg cm\(^2\) dmol\(^{-1}\)) was calculated using the equation \([\theta]=\theta_{\text{obs}}/(n\times l \times c \times 10).\) \(\theta_{\text{obs}}\) is the measured ellipticity in millidegrees, \(n\) is the number of side groups, \(l\) is the path length in centimeters (0.1 cm), and \(c\) is the concentration of the sulfonamido-γ-AAApeptide in molar unit.
4.5.5 **CD spectroscopy for mechanistic study**

The secondary structures of the single peptides N36, or C34, or sequence 12' or the complexes C34/N36, or sequence 12'/N36 were assessed by CD spectroscopy, as described in the previous report. Briefly, N36 and/or CHR-peptide in PBS (10 μM) was incubated at 37 °C for 30 min and then measured on a Jasco spectropolarimeter (Model J-815; Jasco, Inc., Easton, MD), using a 1 nm bandwidth with a 1nm step resolution from 195 to 260 nm at room temperature. The baseline curve was determined on PBS alone.

4.5.6 **Fluorescence polarization assay**

50 nM FITC-labeled 12' were incubated with protein (0-2 μM) in PBS with or without 5 μM C34. Dissociation constants (Kd) were determined by plotting fluorescence anisotropy values as a function of protein concentration, and the plots were fitted to the following equation:

\[
y = [FP_{\text{min}} + (FP_{\text{max}} - FP_{\text{min}})] \frac{(K_d + L_{st} + x) - \sqrt{(K_d + L_{st} + x)^2 - 4L_{st} \times x}}{2L_{st}}
\]

where \(L_{st}\) and \(x\) refer to the concentration of peptide and protein, respectively. The experiments were conducted in triplicate.

4.5.7 **Enzymatic stability study**

Sequence 2, sequence 12 and regular peptide MTSC22EK (0.1 mg/mL) were mixed with protease (0.1 mg/mL) in 100 mM ammonium bicarbonate buffer (pH 7.8) at 37 °C for 24 h. After that, water and ammonium bicarbonate from reaction mixtures were removed by speed vacuum at medium temperature. The residues were dissolved in 100 μL H₂O/CH₃CN and analyzed on a
Waters Alliance HPLC system with a flow rate of 1 mL/min and 5 to 100% linear gradient of solvent B (0.1% TFA in CH₃CN) in A (0.1% TFA in H₂O) over a duration of 50 min.

4.5.8 **PAMPA-BBB assay**

The PAMPA-BBB assay procedure was developed by pION, according to a prior report. The TECAN freedom EVO150 robot was used for all liquid handling operations, and Pion PAMPA Evolution software was used for analysis. Brain the sink buffer (BSB), lipid solution (BBB-1), and stirwell™ PAMPA sandwich plate with magnetic stirring disks were all included in BBB PAMPA. Transferring 4 µL of the lipid solution into the acceptor well, 200 µL of BSB (pH 7.4) were then added. The donor wells were then filled with 180 µL of diluted test chemicals (50-250 µM in system buffer at pH 7.4 from a 10 mM DMSO solution). The PAMPA sandwich plate was put together, set up on the Gut-Box™, and incubated for one hour while being stirred at 60 µm Aqueous Boundary Layer (ABL) settings. The distribution of the sequences in the donor and acceptor buffer (150 µL aliquot) was determined by UV spectra measurement from 250 to 498 nm using the TECAN Infinite M-1000 Pro microplate reader. Permeability (Pₐₚₚ, 10⁻⁶cm/s) of each sequence was calculated by Pion PAMPA evolution software. The assay was performed in triplicate.

4.5.9 **PAMPA-GIT assay**

A technique created by pION was also used to realize the PAMPA-GIT assay. We also used the TECAN freedom EVO150 robot to complete all liquid handling tasks, and pION’s PAMPA Evolution Software was used to evaluate the data. The Stirwell™ PAMPA sandwich plate, preloaded with magnetic disks, the GIT-0 Lipid solution, and the Acceptor Sink Buffer
(ASB) are all components of the pION’s GIT PAMPA. In the acceptor well, 4 μL of lipid were transferred before 200 μL of ASB were added (pH 7.4). The donor wells were then filled with 180 μL of the test substance that had been diluted (50–250 μM in system buffer at pH 5.0, 6.2, and 7.4 from a 10 mM DMSO solution). The Gut-Box™ was filled with the PAMPA sandwich plate, which had been built, and swirled for 40 μm Aqueous Boundary Layer (ABL) settings for 30 min. Distribution of the sequences in the donor and acceptor buffers (150 µL aliquot) was determined by UV spectra measurement from 250 to 498 nm using the TECAN Infinite M-1000 Pro microplate reader. Then the permeability (P app, 10^-6 cm/s) of each sequence was calculated by Pion PAMPA evolution software. The assay was performed in triplicate.

4.5.10 HIV-1 pseudovirus infection assay

The antiviral activity of inhibitors was determined against five replication-competent HIV-1 clinical isolates, as described previously. Briefly, viral stocks were generated by transferring viral molecular clones into HEK293T cells. After transfection for 48 h, virus-containing culture supernatants were harvested, and TCID50 was quantitated in TZM-bl cells. The inhibitor was three-fold diluted, mixed with 100 TCID50 of virus, and added to TZM-bl cells. Luciferase activity of cells was assessed after 48 hours of incubation, and IC50 was subsequently determined.

4.5.11 HIV-1 authentic infection assay

The inhibitory activity of sulfonyl-γ-AApeptides on infection of laboratory-adapted HIV-1 X4 strain IIIB and two HIV-1 clinical isolates was determined, as previously described. 50 μL of a peptide and 50 μL of 100 × TCID50 (50% tissue culture infective doses) HIV-1 authentic virus were mixed for each well of a 96-well plate, and the mixture was then incubated at 37 °C for 30
min. Afterwards, $2 \times 10^4$ MT-2 (for X4 virus) or CEMx174 5.25 M7 cells (for R5 virus) were added. The supernatant was replaced with fresh RPMI-1640 medium containing 10% fetal bovine serum (FBS) after overnight culture. After further culture at 37 °C for three days, 50 µL of the culture medium were collected and mixed with equal volume of 5% (v/v) Triton X-100. ELISA was used to detect p24 antigen, which represents the quantity of HIV-1. Briefly, the collected mixtures were added to a plate coated with anti-HIV-1 Immune Globulin (HIVIG) from the NIH AIDS Reagent Program. Anti-p24 mAb 183, rabbit anti-mouse IgG-HRP (Dako, Glostrup, Denmark) and substrate 3,3,5,5-TMB (Sigma-Aldrich, New York, NY) were added and washed away sequentially. The absorbance at 450 nm (A450) was determined by a Multi-Detection Microplate Reader (Ultra 384, Tecan, Tokyo, Japan). IC$_{50}$s were calculated using Calcusyn software (Biosoft, Ferguson, MO), and the lines of best fit were drawn using GraphPad Prism 8 software (La Jolla, CA).

4.5.12 Cytotoxicity assay

The cytotoxic effects of sulfonyl-γ-AApeptides on MT-2 cells were determined, as previously described.62 The sulfonyl-γ-AApeptide at graded concentration was incubated with $2 \times 10^5$/mL cells at 37 °C for three days before adding 10 µL of CCK8 reagent. After another 2 h incubation at 37 °C, A450 was measured with the Multi-Detection Microplate Reader. Cell viability was calculated by dividing A450 of untreated cells by A450 of cells treated with a sulfonyl-γ-AApeptide.
4.6 $^1$H and $^{13}$C NMR spectra of L-sulfonyl-$\gamma$-AA peptide building blocks

$^1$H NMR of BB10

$^{13}$C NMR of BB10
**1H NMR of BB11**

**13C NMR of BB11**

147
$^{1}H$ NMR of BB12

$^{13}C$ NMR of BB12
$^1$H NMR of BB13

$^{13}$C NMR of BB13
4.7 References


Appendices
Appendix A: Appendix for Figure 1
Appendix B: Appendix for the Introduction
Appendix C: Appendix for Chapter 2

A novel cyclic γ-AApeptide-based long-acting pan-coronavirus fusion inhibitor with potential oral bioavailability by targeting two sites in spike protein

**Author:** Songyi Xue et al  
**Publication:** Cell Discovery  
**Publisher:** Springer Nature  
**Date:** Sep 8, 2022

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