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Sulfono-γ-AA modified peptides that inhibit HIV-1 fusion†

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The utilization of bioactive peptides in the development of highly selective and potent pharmacological agents for the disruption of protein–protein interactions is appealing for drug discovery. It is known that HIV-1 entry into a host cell is through a fusion process that is mediated by the trimeric viral glycoprotein gp120/41, which is derived from gp160 through proteolytic processing. Peptides derived from the HIV gp41 C-terminus have proven to be potent in inhibiting the fusion process. These peptides bind tightly to the hydrophobic pocket on the gp-41 N-terminus, which was previously identified as a potential inhibitor binding site. In this study, we introduce modified 23-residue C-peptides, **3** and **4**, bearing a sulfono-γ-AA residue substitution and hydrocarbon stapling, respectively, which were developed for HIV-1 gp-41 N-terminus binding. Intriguingly, both **3** and **4** were capable of inhibiting envelope-mediated membrane fusion in cell–cell fusion assays at nanomolar potency. Our study reveals that sulfono-γ-AA modified peptides could be used for the development of more potent anti-HIV agents.

Introduction

The global HIV statistics of 2015 revealed that approximately 36.7 million people worldwide are living with the human immunodeficiency virus (HIV) and over 2.1 million new cases of HIV infection were recorded annually.[1,2] During the membrane fusion process, gp41 of HIV forms a fusion-active six-helix bundle (6-HB) with a hydrophobic pocket.[3] In this six-helix bundle, the amino-terminal heptad repeat (N-HR) forms a central trimeric coiled-coil core, whereas the trimeric helical carboxy-terminal heptad repeat (C-HR) wraps around and interacts with it in an antiparallel mode.[4] This six-helix bundle brings the viral and host cell membranes into close proximity and facilitates their fusion.[5] The mutual dependence of N-HR and C-HR on each other in the 6-HB formation during viral entry makes them very important targets for fusion inhibitor design (Fig. 1A). Indeed, targeting the viral entry and fusion process of an enveloped virus remains a very appealing therapeutic strategy due to its relative accessibility. Potent inhibitors which block these specific interactions that are mandatory for HIV-1 viral entry have been reported. For instance, small molecules,[6] engineered peptides[7] and artificially designed peptidomimetics[8] have been developed for the inhibition of viral entry and fusion. However, peptides derived from N-HR mostly show weak inhibitory activities if the design does not promote trimerization of N-HR peptides.[4,9] Only typical N-HR constructs

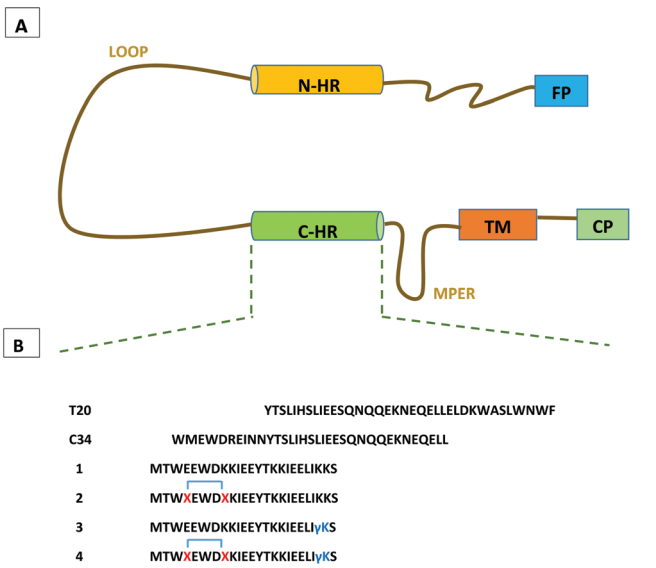

Fig. 1 (A) Schematic illustration of the different regions of HIV-1 gp41. FP: fusion peptide, N-HR: N heptad repeat, C-HR: C heptad repeat, MPER: membrane proximal ectodomain region, TM: transmembrane region, and CP: cytoplasmic domain and (B) C-HR derived peptides. "X" shows the position of the hydrocarbon staple; "γK" is sulfono-γ-AA1. Peptide **1** is MT-SC22EK and was first reported in ref. 16*b*.

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forming stable trimers can efficiently target HIV-1 fusion.[10] As a result, C-HR derived peptides have been studied extensively in the therapeutic search for potential fusion inhibitors. Examples include α-helical peptides such as enfuvirtide (T20)[11] and C34 (Fig. 1B).[12]

Enfuvirtide obtained market approval as the only HIV-1 fusion inhibitor for clinical use and it works by competitively binding the N-HR, thereby blocking the formation of the six-helix bundle required for fusion. It is very active against various HIV-1 strains including those resistant to reverse transcriptase and protease inhibitors.[11b,13] It is similar in design to a segment of C-HR comprising amino acids 127 to 162 of the C-terminal end (Fig. 1B).[14] Although T20 has great anti-HIV activity, it is prone to induce drug resistance through mutations within the N-HR sites. Additionally, its poor bioavailability and large dose requirements complicate its therapeutic use.[15] Similar to T20, C34 also has sequence homology with C-HR. Due to a 22-amino acid overlap between T-20 and C34 peptides, HIV-1 has also developed major mutations for C34 resistance *in vitro*.[15b]

To overcome the problems posed by T20 and other similar HIV fusion inhibitors, great efforts have been made to optimize the fusion inhibitors derived from the C-HR helical region of gp41 in order to suppress the emergence of resistant strains and increase the *in vivo* stability and N-HR binding affinity.[16] One approach is to introduce electrostatic constraints into peptides to improve the helicity and antiviral activity profile. This entails the substitution of charged and hydrophilic amino acids such as glutamic acid (E) and lysine (K) at *i* and *i* + 4 positions in the solvent-accessible site of C34 and its short variants.[3b,17] In a six helix bundle, C-HR interacts with N-HR at the amino acid residues at the *a* and *d* positions of the heptad repeat (Fig. 2A & C). These residues are known to be critical for molecular recognition between both heptad repeats, while residues at positions *b*, *c*, *f* and *g* are exposed to the solution and are almost non-crucial for gp41 C-HR and N-HR interactions (Fig. 2A).[5a] However, the residues at these positions are very critical for solubility and stability and they have great effects on the *in vivo* activity and druggability of the peptide fusion inhibitors. These engineered electrostatic interactions are helix enhancers and have significantly improved the solubility, helicity and potency of such derived peptides.[3b,17a,b,18] Effects on the antiviral activity of the improved helicity of C-peptide variants have been well documented.[17a–c] It is believed that the ability of the derived C-HR peptides to adopt stable helical conformation upon interaction with the N-HR fosters the formation of the six helix bundle (6-HB) by increasing the binding affinity and *in vivo* stability. This in turn increases the anti-HIV activity.[17c]

An additional approach is to introduce conformational restraints by substitution with non-proteinogenic amino acids at the solvent-exposed site of C-HR derived peptide mimics. For instance, peptidomimetics with unnatural amino acids and building blocks have been reported to successfully mimic the molecular interaction between gp41 C-HR and N-HR.[8,14b,16b,c,19] Peptide mimics that target gp41 N-HR include

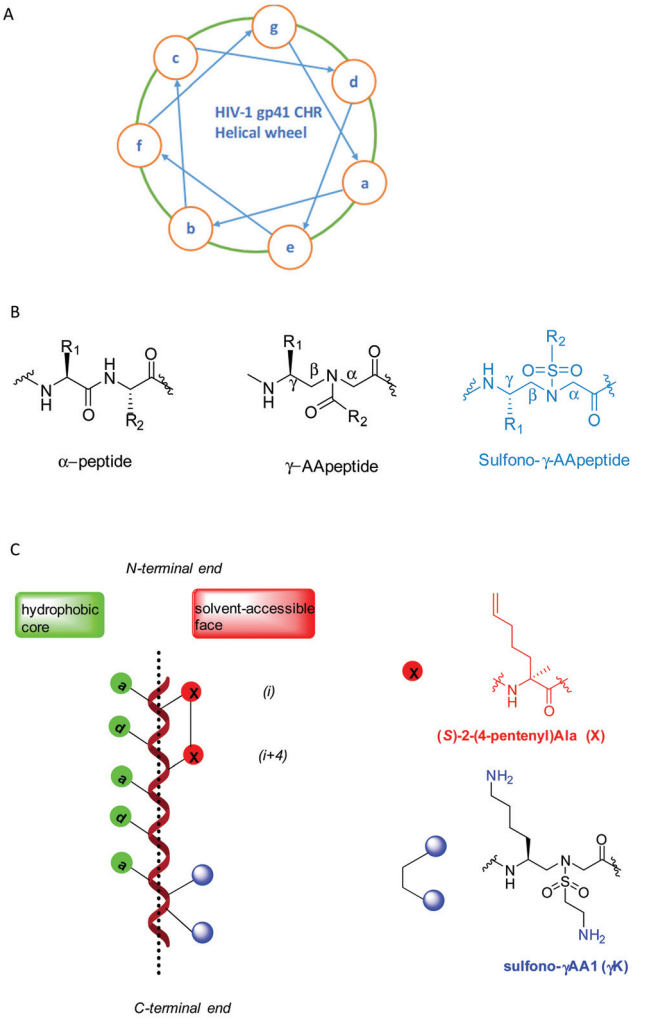

Fig. 2 A. Schematic illustration of the helical wheel of the C-terminal heptad repeat of gp-41. B. General structures of α-peptides, γ-AApeptides and sulfono-γ-AApeptides. C. Schematic illustration of the positioning of the hydrocarbon staple and sulfono-γ-AA1.

D-peptides,[8d,10a] unnatural foldamers,[20] and covalently restrained α-helices.[8b,c,21] These peptide mimics have generated highly potent fusion inhibitors with outstanding *in vivo* stabilities.

Sulfono-γ-AApeptides are a sub-class of γ-AApeptides that are oligomers of *N*-acylated-*N*-aminoethyl amino acids.[22] The replacement of carboxylic acids with sulfonyl chlorides in γ-AApeptides produces sulfono-γ-AApeptides (Fig. 2B). They have enormous potential in functional group diversity. Like γ-AApeptides, sulfono-γ-AApeptides are able to display the same number of side chains as conventional peptides of equal length, endowing them with the ability to mimic bioactive peptides. As evidenced by their crystal structures, sulfono-γ-AApeptides possess an intrinsic folding propensity which is most likely a result of the bulkiness of the tertiary sulfonamide group and intramolecular hydrogen bonding.[23] Their optical analysis by circular dichroism and 2D-NMR also supports their well-crafted helical conformation.[23] To explore the potential of

sulfono-γ-AApeptides for their ability to modulate HIV-1 fusion at the cell entry stage, we designed two peptides (**3** & **4**) containing sulfono-γ-AA residues. Our previous findings suggested that replacement of amino acid residues with sulfono-γ-AA residues could retain sequence helicity.[24] Thus, we envisioned that these sequences were still capable of mimicking gp41.

Results and discussion

Peptide **1** is a 24-residue electrostatically constrained C-HR derived peptide with the MT-hook structure (Table 1), which was previously reported to be an active fusion inhibitor.[16b] Thus, it was used as the template sequence for modification (Fig. 1B).[16b] At first, we substituted all the "EE" and "KK"-motifs in peptide **1** with "**γE**" and "**γK**", respectively (Table S2 and Fig. S4†). The resulting peptide, **P1**, exhibited poor helicity and antiviral activity (Table S2 and Fig. S5†). We hypothesized that substitution of too many amino acid residues with sulfono-γ-AA residues disrupts the secondary structure of **1**. This led us to limit our point of substitution to either the N- or the C-terminal end, resulting in peptides **P3** and **P4**. Similar to **P1**, **P3** and **P4** displayed poor antiviral activities and helicities (Table S2, Fig. S5†). We believed that inserting both "**γE**" and "**γK**" in the same sequence could sufficiently affect the folding propensity of **1**. We then decided to study the effects of "**γK**" substitution on the helicity and antiviral activity of **1**. We designed peptides **3** (Fig. 1B) and **P2** (Table S2†) with "**γK**" substitution at various points on the C-terminal end. To our surprise, **3** showed similar antiviral activity to **1** (Table 2). However, **P2** displayed poor antiviral activity and poor helical propensity (Table S2, Fig. S5†). As such, our attention was paid to the development of **3**.

In order to optimize our design, we sought to improve the helicity of **3** by employing a combination of sulfono-γ-AA residue substitution (**γK**) and single hydrocarbon stapling (Fig. 1B and Table 1). We further developed **2** (control) and **4**. **2** and **4** are stapled variants of **1** and **3**, respectively. **3** contains a sulfono-γ-AA1 residue (**γK**) at the 22nd position, while **2** and **4** contain hydrocarbon staples between residues X at the 4th and 8th positions (Fig. 2C and Table 1). Our decision to install the hydrocarbon staple at the N-terminus was inspired by a previous report by Bird *et al.* They ranked the order of antiviral activities of the hydrocarbon stapled lengthy peptides as follows: double stapling > N-terminus single stapling > C-terminus single stapling.[21]

We next first conducted CD studies to assess the helical propensities of the sequences. As shown in Fig. 3A, both **1** and **3** exhibited comparable helical folding propensities with characteristic minima at both 207 and 222 nm suggesting the existence of probable α-helical conformation. This suggests that incorporation of sulfono-γ-AA residue did not alter helicity significantly. As expected, after hydrocarbon stapling, both **2** and **4** clearly showed a significant increase in helicity compared to their unstapled counterparts, **1** and **3** (Fig. 3A). This is consistent with the previous findings that *i*, *i* + 4 hydrocarbon stapling enhances the α-helicity of peptides.[21,25,26] These findings are corroborated by thermal stability studies (Fig. 3B). **1** and **2** showed similar behavior at high temperature. However, **1** is more stable than **2** at temperatures lower than 35 °C. Also, **4** demonstrated increased stability than **3**. Overall, hydrocarbon stapling improved the helicities and thermal stability profiles of **2** and **4**.

We then examined the antiviral activities of these sulfono-γ-AA containing peptides by a HIV neutralization assay (Table 2).[27]

Conformationally stabilized pre-formed recombinant *env* trimers derived from various subtypes of HIV-1 strains were used in the assay.[28] Both (enfuvirtide) T20 and AZT were also tested for comparison. Interestingly, both **3** and **4** displayed similar antiviral activities across the various strains tested. **1** and **2** also showed a similar trend in antiviral activity. However, **3** displayed increased antiviral activity (<2-fold) than **1**. This indicates that the introduction of the peptidomimetic monomer, sulfono-γ-AA1, slightly improved the interaction of peptide **3** with the N-HR of gp41, resulting in the inhibition of six-helix bundle formation. It is worth noting that antiviral activity is not strictly dependent on secondary structures as evidenced by the obtained results (Table 2), as **3** and **4** exhibited comparable antiviral activities, just like **1** and **2**. All tested peptides showed comparable or greater anti-HIV activities

Table 1 List of peptides and modifications

Peptide	Sequence	Modification
1	MTWEEWDKKIEEYTKKIEELIKKS	—
2	MTWXEWDXKIEEYTKKIEELIKKS	Hydrocarbon stapling
3	MTWEEWDKKIEEYTKKIEELIγKS	Sulfono-γ-AA1 (γK)
4	MTWXEWDXKIEEYTKKIEELIγKS	Both

Table 2 Antiviral activity from the HIV neutralisation assay

IC_{50}[a] (nM)	CZA97	B41	BG505	SF162	MN	DU422
1	6.8 ± 1.6	181.1 ± 89.1	15.2 ± 0.2	11.3 ± 2.2	102.4 ± 29.9	14.8 ± 0.3
2	7.6 ± 1.0	210.8 ± 58.9	14.0 ± 0.5	15.6 ± 0.9	67.1 ± 9.1	16.0 ± 1.1
3	4.6 ± 0.1	118.8 ± 4.0	9.0 ± 0.2	7.2 ± 1.8	77.9 ± 2.4	6.0 ± 2.7
4	6.5 ± 0.8	171.3 ± 4.6	9.7 ± 2.6	11.4 ± 0.8	51.2 ± 10.2	11.3 ± 0.1
T20	941.0 ± 328.3	214.6 ± 16.2	18.4 ± 10.1	23.9 ± 2.1	6.5 ± 0.6	56.0 ± 1.6
AZT	184.6 ± 26.4	106.4 ± 16.8	84.0 ± 1.8	109.6 ± 23.5	194.1 ± 1.6	183.3 ± 1.9

[a] Antiviral activity shown as the IC_{50} was determined by the HIV neutralization assay. Each IC_{50} value represents the mean ± SEM obtained from at least two independent experiments.

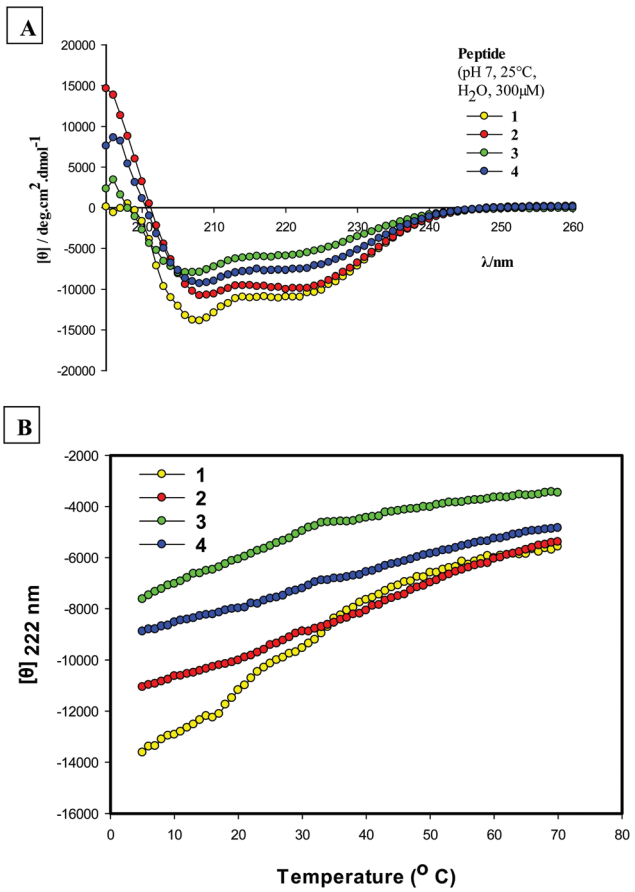

Fig. 3 (A) CD spectra of peptides **1–4**. (B) Change in mean residue ellipticity $[\theta]_{222nm}$ for peptides **1–4** as a function of temperature (5–70 °C). Conditions – pH 7, 25 °C, solvent – H_2O.

than enfuvirtide (T20), a fusion inhibitor, and AZT, a reverse transcriptase inhibitor, which are currently being used in the clinics for the treatment of the symptomatic disease (Table 2). Thus, **3** emerged to be the most active peptide with the best antiviral activity across all strains tested (<2-fold increase).

To directly interrogate the effect of sulfono-γ-AA1 substitution and hydrocarbon stapling on proteolytic cleavage, we used LC/MS to identify proteolytic fragments generated by the digestion of our peptide panels with chymotrypsin. After 10 min, **1**, **2** and **4** demonstrated greater stabilities to chymotrypsin than **3** (Table 3 and Fig. S6†), suggesting that the inclusion of the sulfono-γ-AA residue slightly altered the helicity, making it easier to be hydrolyzed. However, after backbone stapling, still possessing one sulfono-γ-AA residue, **4** exhibited almost similar resistance to proteolytic degradation as **1**, indicating that the introduction of unnatural residues could enhance the stability of the sequence.

Table 3 Enzymatic hydrolysis assay with chymotrypsin

Peptide	% remaining after 10 min
1	53.3
2	69.3
3	17.6
4	57.2

Conclusions

To summarize, although the daunting challenges hampering the clinical applications of enfuvirtide (T20) have limited the repository of active peptide-fusion inhibitors, the possibility of incorporating non-natural scaffolds may ultimately usher in a new generation of peptide-fusion inhibitors with great therapeutic potential and improved protease resistance. Although our current design did not yield peptides with enhanced stabilities toward proteolysis, our study reveals that sulfono-γ-AA modified peptides could be used for the development of more potent anti-HIV agents. Furthermore, it is known that homogeneous sulfono-γ-AApeptides are completely resistant to enzymatic degradation and they possess remarkable helical propensities. We envision that we could use homogeneous sulfono-γ-AApeptides to design HIV-1 gp41 mimetics in the future. Additionally, the strategy implemented herein not only provides ideas for future HIV-1 inhibitor design, but may also be explored for protein–protein interaction inhibitor (PPII) design.

Conflicts of interest

There are no conflicts to declare in this work.

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