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Design of antiviral stapled peptides containing a biphenyl cross-linker

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Abstract

Here we report the design and synthesis of a panel of stapled peptides containing a distance-matching biphenyl cross-linker based upon a peptide capsid assembly inhibitor reported previously. Compared with the linear peptide, the biphenyl-stapled peptides exhibited significantly enhanced cell penetration and potent antiviral activity in the cell-based infection assays. Isothermal titration calorimetry and surface plasmon resonance experiments revealed that the most active stapled CAI peptide binds to the C-terminal domain of HIV capsid protein as well as envelop glycoprotein gp120 with low micromolar binding affinities, and as a result, inhibits both the HIV-1 virus entry and the virus assembly.

Keywords

peptides; protein-protein interaction; HIV capsid; virus assembly; virus entry

Helix-mediated protein-protein interactions are attractive drug targets because they mediate diverse fundamentally important biological processes including cell growth, apoptosis, and virus assembly.¹ For example, a phage-based selection has led to the discovery of a peptide capsid assembly inhibitor (CAI; sequence = ITFEDLLDYYGP) that binds to the C-terminal domain (CTD) of the HIV capsid protein p24 with a K_d of 15 μ M.² Since p24 is responsible for the oligomerization and assembly of protein envelope surrounding virus genome, the binding of helical CAI to CTD inhibits the assembly of immature HIV-1.³ Due to the lack of cell penetration, however, CAI peptide failed to show antiviral activities in the cell-based assays. To overcome this problem, we previously reported the use of hydrocarbon stapling chemistry⁴ to convert linear CAI peptide into a cell-penetrating stapled peptide that showed

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Supplemental tables and experimental procedures.

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potent and broad antiviral activities.⁵ In an effort to use simpler stapling chemistries to generate cell-permeable CAI, herein we report the synthesis of stapled CAI peptides using a cysteine-based biaryl cross-linking chemistry and characterization of their antiviral activities in the cell-based single-cycle and multi-cycle infection assays. Furthermore, we found these biaryl-stapled CAI peptides exhibited dual activities in inhibiting both the virus assembly and the virus entry by binding to the C-terminal domain of the capsid protein and the envelop glycoprotein gp120, respectively.

We recently reported the design of distance-matching biaryl cross-linkers suitable for stapling peptides containing cysteines at *i,i*+7 positions.⁶ The applications of this stapling chemistry to bioactive peptides have led to increased biological activity, improved cell penetration, and enhanced serum stability.⁷ To apply biaryl stapling chemistry to CAI, we needed to replace two solvent-exposed residues in CAI with two cysteines. Inspection of the crystal structure of the CAI-CTD complex revealed that the CAI peptide inserts into the deep hydrophobic groove of CTD as an amphipathic α -helix, with the hydrophobic face composed of Phe-3, Leu-6, Leu-7, Tyr-9 and Tyr-10 making van der Waals interaction with the CTD hydrophobic groove (Figure 1). To preserve the hydrophobic contact, we replaced the solvent exposed Glu-4 and Gly-11 with D- and L-cysteine, respectively, to prepare for the installation of the biphenyl cross-linker, Bph (Figure 1b). Previously, we showed that the substitution of Pro-12 with Glu at the C terminus was beneficial as Glu forms a salt bridge with the Arg173 in the CTD. Since CAI is comprised of more than 50% hydrophobic residues, we further appended a Ser-Gly-Ser tail at the C terminus in order to enhance water solubility. A series of Bph-stapled CAI peptides were thus prepared (Table 1, see the Supplemental Information for details). Substitutions were then made at the selected positions in order to probe the importance of the individual residues. The identities of the stapled CAI peptides were confirmed by mass spectrometry (Table S1).

To examine whether Bph-stapling leads to increased antiviral activity, we evaluated the stapled CAI peptides in both multi-cycle infection assay and single-cycle neutralization assay using MT-2 and TZM-bl cells, respectively. All stapled peptides exhibited low-micromolar potency ($IC_{50} < 10 \mu M$) with the exception of stapled peptide **6** in which Tyr-10 is replaced by Ala (Table 1). Because Tyr-10 is a key hydrophobic residue involved in the binding to CTD, it is expected that substitution with Ala will result in reduced binding and significant reduction in antiviral activity. Compared to peptide **1**, peptides **2–5** with substitutions of Glu-5 by Gln, Leu-7 by Ala, and/or Asp-8 by Asn resulted in modest decreases in antiviral activities (Table 1), likely due to their peripheral locations with respect to the binding interface (Figure 1b). In contrast, linear CAI peptide was inactive in both multi- and single-cycle assays, indicating that Bph-stapling is both necessary and sufficient in generating potent antiviral activities. Among the peptides tested, peptide **1** gave the highest antiviral activity in both assays (Table 1). Importantly, we did not observe any toxicity at the peptide concentrations used in the cell-based assays ($CC_{50} > 100 \mu M$ for majority of the stapled peptides in both cell lines, see Table S2 for details), indicating that the reduction in p24 levels (multi-cycle) or luciferase production (single-cycle) in cells treated with the stapled peptides is due to viral inhibition and not to cytotoxicity.

Subsequently, we tested stapled peptide **1** that shows the highest antiviral activity and selectivity index ($SI = CC_{50}/IC_{50}$) against a panel of pseudo viruses pseudotyped with ENVs from primary HIV-1 isolates subtype A, A/D, A/G, B, C and D reference viruses in the single-cycle assay. These Env-pseudotyped viruses exhibit a neutralization phenotype that is typical of most primary HIV-1 isolates.⁹ As shown in Table 2, peptide **1** is active against all subtypes of HIV-1 viruses ($IC_{50} = 1.0\sim 17.9\ \mu M$). In particular, peptide **1** was most effective against the viruses from subtype B with IC_{50} values in the range of $1.6\sim 4.6\ \mu M$. Since all the clones share the same CTD but differ in the envelopes (ENVs), the discrete sensitivity is likely due to the difference in interacting with the envelope of HIV-1. However, in the absence of the knowledge about the binding site for the stapled peptide, we analyzed the entire gp160 sequences of all the clones and could not identify the specific patterns underlining higher sensitivity of some clones over the others.

To confirm that Bph stapling increases cell-penetration, we further prepared a FITC-labeled stapled peptide **1**, and compared its uptake by HEK293T cells to FITC-labeled CAI. Figure 2 shows that cells treated with FITC-**1** showed strong intracellular fluorescence compared to cells treated with FITC-CAI, indicating that Bph-stapling is responsible for the cellular uptake of the peptide. The increased cell penetration is likely due to the increased hydrophobicity as the far-UV CD measurement did not show increased helicity for stapled peptide **1** compared to linear CAI peptide (data not shown).

Since peptide **1** exhibited the highest activity in inhibiting the virus assembly as indicated in multi-cycle assay, we measured the binding affinity of peptide **1** toward the C-terminal domain (CTD) of the HIV capsid protein using isothermal titration calorimetry (ITC) (Figure 3). The integrated binding isotherm curve was fitted to the single site binding model to yield the peptide **1**:CTD binding stoichiometry of 1.35 ± 0.185 along with the thermodynamic parameters $H = 1.431 \pm 0.259\ \text{kcal/mol}$, $S = 18.4\ \text{cal/mol}\cdot\text{K}$, and dissociation constant $K_d = 17.5 \pm 6.2\ \mu M$. In contrast, the ITC measurement with the linear CAI peptide gave the CAI:CTD binding stoichiometry of 0.98 ± 0.05 , $H = -9.45 \pm 0.64\ \text{kcal/mol}$, $S = -7.91\ \text{cal/mol}\cdot\text{K}$, and $K_d = 8.3 \pm 0.71\ \mu M$. Therefore, despite a two-fold reduction in binding affinity toward CTD compared to linear CAI, stapled peptide **1** exhibited greater inhibition of the virus assembly, which can be attributed to its significantly improved cell-penetration (Figure 2).

Since stapled CAI peptides showed higher potency in the single-cycle virus neutralization assay than in the multi-cycle virus infection assay, we suspected that these peptides may also inhibit HIV-1 virus entry in addition to virus assembly. To probe this possibility, we examined whether peptide **1** can bind to HIV-1 envelope glycoprotein gp120 as many of the inhibitors that target the early stage of virus life cycle bind to the HIV-1 envelope glycoprotein.^{8,10} We employed the surface plasmon resonance (SPR) technique to determine the binding affinity of peptide **1** towards the Yu2gp120 protein (Figure 4). By fitting the resulting sensorgrams to the simple 1:1 (Langmuir) binding model, the association rate constant (k_a) was determined to be $7.44 \times 10^2\ \text{M}^{-1}\ \text{s}^{-1}$ while the dissociation rate constant was determined to be $5.53 \times 10^{-3}\ \text{s}^{-1}$. The dissociation constant, K_d , was then calculated to be $7.4\ \mu M$, which is surprisingly lower than the dissociation constant against CTD ($K_d = 17.5 \pm 6.2\ \mu M$) as measured by ITC (Figure 3), suggesting that the stapled CAI peptide can

impede HIV-1 virus entry through an unexpected binding to the envelop glycoprotein. Since gp120 does not share sequence or structure homology with the CTD of p24, it remains to be determined to which region of gp120 the stapled peptide 1 binds. Similar inhibition of HIV-1 virus entry was also observed with the hydrocarbon stapled CAI peptides such as NYAD-36 (Table 1).⁸

In summary, we have successfully extended the cysteine-based biaryl stapling chemistry to the design of cell-permeable, stapled CAI peptides that showed potent and broad antiviral activities. ITC and SPR-based mechanistic studies indicated that the most potent stapled CAI peptide inhibits HIV-1 virus entry by binding to the envelop glycoprotein gp120 as well as the virus assembly by binding to the C-terminal domain of the HIV-1 capsid protein. Since the stapled peptide **1** only showed modest binding affinities toward CTD ($K_d = 17.5 \mu\text{M}$) and gp120 ($K_d = 7.4 \mu\text{M}$), the enhanced activity observed in the cell-based assays ($\text{IC}_{50} = 1\text{--}2 \mu\text{M}$) suggests that the binding of these two targets has synergistic effect on inhibiting HIV virus replication. This unique dual-binding mode is particularly attractive for designing potent and selective antiviral therapies targeting the multiple steps of the HIV-1 life cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Crystal structure of CAI bound to CTD (PDB code: 2BUO). CTD is rendered in surface model while CAI is shown in ribbon. The side chains of hydrophobic residues F3, L6, L7, Y9 and Y10 are shown in blue tube while the solvent exposed hydrophilic residues are shown in green tube. (b) Helical wheel diagram of CAI viewed from the N- to C-terminus. The residues suitable for stapling are colored in red, and the chemical structure of Bph is shown.

Figure 2.

Comparison of the cell-penetration property of CAI and stapled peptide **1** in HEK293T cells. Representative confocal microscopy images of HEK293T cells incubated with 5 μ M of FITC-labeled peptide at 37°C for 2 h. DIC = differential interference contrast; scale bar = 20 μ m.

Figure 3.

Isothermal titration calorimetric analyses of the CTDM184A-W185A-peptide interactions. (a) 625 μM CTDM184A/W185A was titrated in 10 μL aliquots into 20 μM peptide 1. (b) 350 μM CTDM184A/W185A was titrated in 10 μL aliquots into 15 μM CAI. Integrated binding isotherms of the titration (bottom) are fitted to a single-site binding equation.

Figure 4.

Kinetic analysis of stapled peptide **1**/gp120 interaction by surface plasmon resonance (SPR). Kinetic data were collected for different concentrations of peptide **1** to a gp120 surface. Yu2 full-length gp120 was immobilized on a CM5 sensor chip, and 2-fold dilutions of peptide **1** were allowed to pass over the sensor chip.

Table 1

Sequences of CAI peptides and their antiviral activities in multi-cycle (MT-2) and single-cycle (TZM-b1) assays ^a

peptide	sequence	MT-2 cells IC ₅₀ (μM)	TZM-b1 cells IC ₅₀ (μM)
CAI	ITFEDLLDYYP	>135	>135
NYAD-36 ^b	ISFR ₈ ELLDYYS ₅ ESGS ^c	1.5 ± 0.17	2.0 ± 0.4
1	ISFc'ELLDYYC'ESGS	2.8 ± 0.2	1.9 ± 0.2
2	ISFc'QLLDYYC'ESGS ^d	5.5 ± 1.4	3.1 ± 0.3
3	ISFc'ELLDYYC'ESGS	8.8 ± 0.5	1.8 ± 0.1
4	ISFc'QLLDYYC'ESGS	5.7 ± 0.1	1.6 ± 0.2
5	ISFc'ELADYYC'ESGS	4.1 ± 0.3	1.4 ± 0.2
6	ISFc'ELLDYAC'ESGS	14.0 ± 1.1	17.3 ± 1.4

^aThe antiviral assays were performed three times to obtain average IC₅₀ ± SD.

^bActivity data were drawn from reference 8.

^cHydrocarbon stapling occurs between R₈ and S₅: R₈ = (R)-2-(7'-octenyl)alanine; S₅ = (S)-2-(4'-pentenyl)alanine.

^dc' and C' denote Bph-linked D-cysteine and L-cysteine, respectively.

NA, no activity; ND, not determined

Table 2

Neutralization activity of stapled peptide **1** in single-cycle assay against a panel of HIV-1 ENV pseudotyped reference viruses

NIH #	ENV clone	Subtype	IC ₅₀ (μM)
11889	QB726.70M.ENV.B3	A	~15.4
11891	QF495.23M.ENV.A3	A	1.4 ± 0.06
11892	QF495.23M.ENV.B2	A	~10
11896	QH343.21M.ENV.A10	A	~15.4
11901	QA790.204I.ENV.A4	AD	7.0 ± 0.3
11904	QA790.204I.ENV.E2	AD	~7.7
11601	CRF02_AG (Clone 263)	AG	4.4 ± 0.25
11591	CRF02_AG (Clone 211)	AG	4.0 ± 0.7
11035	pREJO4541 clone 67 (SVPB16)	B	3.4 ± 0.5
11036	pRHPA4259 clone 7 (SVPB14)	B	1.6 ± 0.3
11038	pCAAN5342 clone A2 (SVPB19)	B	4.6 ± 0.5
11022	PVO, clone 4 (SVPB11)	B	3.5 ± 0.1
11058	SC422661, clone B (SVPB8)	B	3.7 ± 0.6
11306	Du156, clone 12 (SVPC3)	C	17.9 ± 1.7
11311	ZM233M.PB6, SVPC9	C	3.6 ± 0.5
11315	ZM135M.PL10a, SVPC15	C	1.0 ± 0.2
11507	HIV-25925-2, clone 22	C	1.1 ± 0.2
11508	HIV-26191-2, clone 48	C	6.3 ± 0.1
11912	QA013.70I.ENV.M12	D	~15.4
11913	QA465.59M.ENV.A1	D	~15.4
11914	QA465.59M.ENV.D1	D	~7.7
11916	QD435.100M.ENV.B5	D	~15.4
11917	QD435.100M.ENV.A4	D	1.0 ± 0.3
11918	QD435.100M.ENV.E1	D	~15.4