Design of a Novel HIV-1 Fusion Inhibitor That Displays a Minimal Interface for Binding Affinity

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Abstract: Reported herein are the design, biological activities, and biophysical properties of a novel HIV-1 membrane fusion inhibitor. α-Helix-inducible X-EE-XX-KK motifs were applied to design an enfuvirtide analogue **2** that exhibited highly potent anti-HIV activity against wild-type HIV-1, enfuvirtide-resistant HIV-1 strains, and an HIV-2 strain in vitro. Indispensable residues for bioactivity of enfuvirtide, including the residues interacting with the N-terminal heptad repeat and the C-terminal hydrophobic residues, were identified.

The viral entry process of human immunodeficiency virus type 1 (HIV-1[a]) into target cells is mediated by envelope glycoprotein gp41. Formation of a fusogenic six-helical bundle structure consisting of a gp41 N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR) promotes the fusion of viral and cellular membranes (Figure 1a).[1] Enfuvirtide **1** (T-20, DP178) is an approved anti-HIV peptide derived from the gp41 CHR.[2,3] This first drug that inhibits HIV-1 entry into the cell is utilized as an alternative anti-HIV agent for patients with drug resistance to reverse transcriptase and/or protease inhibitors. It is believed that peptide **1** interacts with the NHR of gp41 prehairpin structure[4] and associates with the cell or viral membrane through a C-terminal tryptophan-rich region,[5–7] but the exact action mechanism of **1** has not been clarified.[8,9]

Stabilization of bioactive conformations of peptides is a promising approach to enhance their biological potency and to understand the bioactive conformation. Several approaches to stabilize the α-helix structure of gp41 CHR have been reported including macrocyclization by covalent bond formation[10] or salt-bridge formation[11–13] between two adjacent residues and/or introduction of α-helix-inducible peptide sequences.[11] The analogue of another CHR peptide C34, in which the residues on the outer surface of the six-helical bundle were comprehensively replaced with glutamates (Glu) or lysines (Lys), retained highly potent anti-HIV activity.[12] This indicates that the substituted residues are not associated with an NHR coiled-coil as suggested by the crystal structure of the N36-C34 complex.[14] Our expectation was that the following three functional surfaces of **1** could be characterized by extending this molecular design (Figure 1b): (1) minimal interface residues for affinity with NHR; (2) solvent-accessible sites to be utilized for α-helix inducible salt bridges; (3) another functional region outside the α-helix structure. Accordingly, efforts herein were undertaken to design novel amphiphilic enfuvirtide derivatives bearing α-helix-inducible motifs.

A schematic wheel of the potential α-helix structure of peptide **1** is depicted in Figure 1c. To introduce salt bridges between i and $i + 4$ residues on the basis of the previous C34 modification,[12] Glu and Lys were arranged at b/c and f/g positions, respectively, so that four consecutive X-EE-XX-KK motifs appeared in the designed peptide **2** (designated T-20EK, Figure 2). All peptides were prepared by standard Fmoc-based peptide synthesis protocol. After final deprotection and cleavage from the resins using a TFA/thioanisole/*m*-cresol/ethanedithiol/H_2O (80:5:5:5:5) cocktail, the crude peptides were purified by reverse-phase HPLC to yield the expected peptides, which were characterized by mass spectrometry. Anti-HIV activity of the peptides against laboratory HIV-1 NL4-3 strain (wild-type) was evaluated by MAGI assay (Table 1). Peptide **2** exhibited 8-fold greater anti-HIV activity compared with the parent peptide **1** [peptide **1**, $EC_{50} = 15 \pm 3.9$ nM; peptide **2**, $EC_{50} = 1.8 \pm 0.4$ nM].[15] The circular dichroism (CD) spectrum of **2** in phosphate buffered saline (pH 7.4) had negative minima at 208 and 222 nm, indicating the presence of an α-helical conformation, while that of **1** suggested a random-coil conformation (Figure 3a). The significant increase in anti-HIV activity of **2** could be rationalized by the preordered stable α-helical structure upstream of L158.[16]

Systematic substitutions of the amino acids at the b, c, f, and g positions with Glu or Lys were extended (Figure 2). Peptide **3**, in which L130 and N160 were substituted with Lys and Glu, respectively, showed anti-HIV activity similar to that of peptide **2** (peptide **3**, $EC_{50} = 2.8 \pm 0.6$ nM). This is consistent with the fact that potent T-1249 also contains these two substitutions.[17] Further replacement toward the N-terminal f position (S129) was again permissive of the high anti-HIV activity (peptide **4**, $EC_{50} = 2.5 \pm 0.6$ nM). On the other hand, replacement of W161 with Glu resulted in a significant decrease of anti-HIV activity as observed in peptides **5** and **6** (EC_{50} = 185 and 111 nM, respectively), indicating that W161 may be located outside the amphiphilic α-helical region. This result correlates with the reduced entry ability of W161F mutant virus.[18] Alanine substitution of W161 also supports the relevance of this residue in virus infectivity and in the inhibitory activity of **1**. Although peptide **7**, carrying a W155A substitution, expressed anti-HIV activity similar to that of peptide **2** (peptide **7**, $EC_{50} = 5.8 \pm 1.0$ nM), W159A, W161A, and F162A substitutions showed reduced bioactivity (peptides **8**, **9**, and **10**, EC_{50} = 49, 24, and 27 nM, respectively). The observed similar CD spectra among peptides **2** and **7–10** demonstrated that alanine substitution had no effect on the stabilized secondary structure of the α-helix (Figure 3b). That is, the hydrophobic indole and phenyl groups of these peptides may contribute to their direct interaction with virus components such as gp41 NHR or the virus membrane.[19]

The comparative binding affinity of peptides **1** and **2** with the gp41 NHR sequence was investigated by pull-down assay using synthetic His-tagged CHR peptides and recombinant MBP-fused NHR protein (Figure 4a). Peptide **2** showed higher affinity with NHR compared with **1**. In contrast, only weak binding was observed in the same experiment using all-D-T-20EK D-**2**, which consists of all D-amino acids, indicating that

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[a] Abbreviations: HIV-1, human immunodeficiency virus type 1; NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat.

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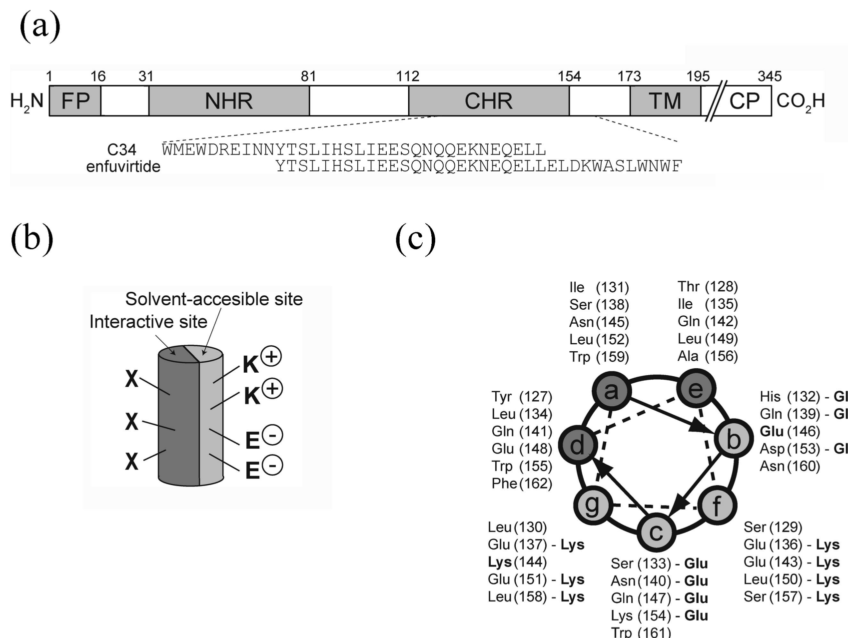

Figure 1. Design of enfuvirtide analogues: (a) schematic representation of HIV-1 gp41 (FP, fusion peptide; NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; TM, transmembrane domain); (b) estimated preordered α-helix structure of CHR peptide by potential salt-bridge formation; (c) helical-wheel representation of peptides **1** and **2**. For peptide **2**, Glu residues are in b and c positions and Lys residues in f and g positions. Residues are numbered starting at the first amino acid of the NL4-3 gp41.

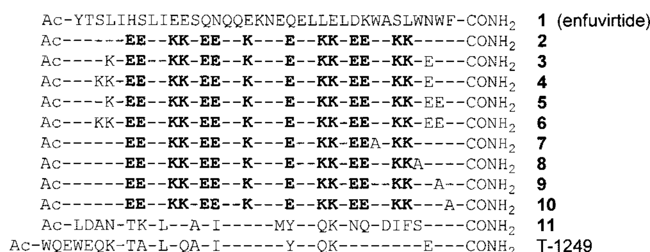

Figure 2. Peptide sequences of enfuvirtide, enfuvirtide analogues, and T-1249.

Table 1. Anti-HIV Activity of Synthetic Enfuvirtide Analogues

peptide	EC_{50} (nM)[a]	peptide	EC_{50} (nM)[a]
1 (enfuvirtide)	15 ± 3.9	**7**	5.8 ± 1.0
2[b]	1.8 ± 0.4	**8**	49 ± 8.6
3	2.8 ± 0.6	**9**	24 ± 3.8
4	2.5 ± 0.6	**10**	27 ± 6.8
5	185 ± 17	C34	4.5 ± 0.5
6	111 ± 25		

[a] EC_{50} was determined as the concentration that blocked HIV-1 replication by 50% in MAGI assay. [b] The enantiomer of peptide **2** (D-**2**) did not show anti-HIV activity at 10 μM.

the binding between **2** and NHR is specific. In addition, peptide **2** inhibited the interaction between **1** and NHR in lower concentration in the inhibition experiment (Figure 4b).[20]

We next evaluated the anti-HIV activity of peptide **2** against enfuvirtide-resistant variants HIV-1_{V38A} and HIV-1_{N43D}, which were mainly isolated from patients resistant to enfuvirtide (Table 2).[21] Because of the deficient replication of these variants,[22] an additional D36G mutation was experimentally added to these variants and to the wild-type virus. The D36G mutation is not involved in enfuvirtide resistance, but it did improve the sensitivity against **1** [EC_{50}(HIV-1_{D36G}) = 2.3 nM] compared with wild-type HIV-1. As reported previously,[21] V38A and N43D mutations significantly reduced the potency of **1** [EC_{50} (HIV-1_{V38A}) = 22 nM; EC_{50}(HIV-1_{N43D}) = 46 nM]. On the other hand, peptide **2** retained similar anti-HIV activity against N43D and slightly less potent activity toward V38A variants [EC_{50}(HIV-1_{V38A}) = 3.3 nM; EC_{50}(HIV-1_{N43D}) = 1.7 nM]. It is of interest that the anti-HIV activity was restored by induction of a bioactive α-helix structure using X-EE-XX-KK motifs on a CHR peptide. This implies that the stable α-helical structure of **2** can overcome the reduced affinity derived from the mismatched interaction between mutated NHR sequences and peptide **1**.

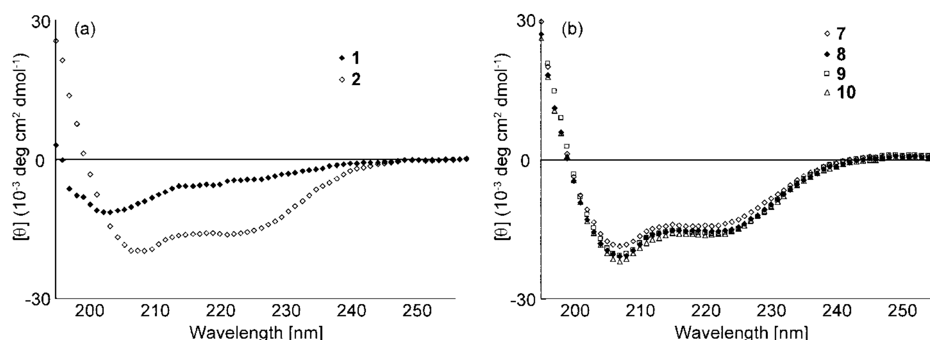

Figure 3. Circular dichroism spectra of (a) peptides **1** and **2** and (b) Ala-substituted peptides **7**–**10**.

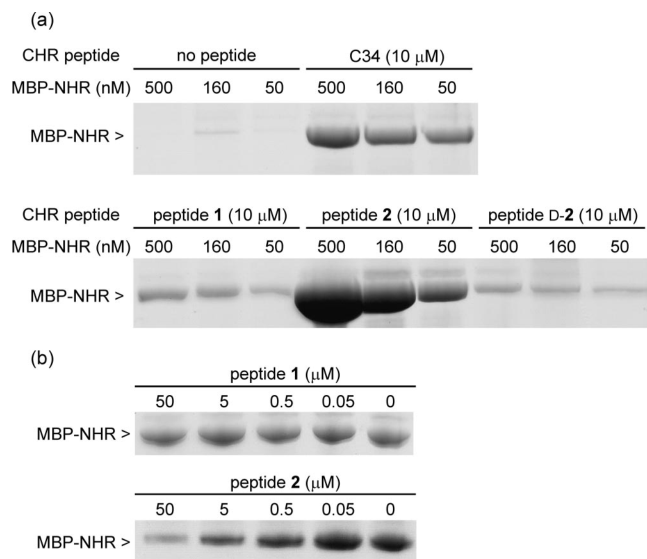

Figure 4. Interaction of His-tagged CHR peptides with MBP-NHR protein by pull-down assay and SDS−PAGE: (a) NHR protein binding with several His-tagged CHR peptides; (b) inhibition of interaction between His-tagged enfuvirtide and NHR protein by nontagged peptides **1** and **2**.

Table 2. Anti-HIV Activity of Peptides **1** and **2** against Enfuvirtide-Resistant Variant and HIV-2 Strains

	EC_{50} (nM)[a]	
strains	peptide **1**	peptide **2**
HIV-1		
NL4–3	15 ± 3.9	1.8 ± 0.4
D36G	2.3 ± 0.5	0.9 ± 0.2
D36G V38A	22 ± 7.6	3.3 ± 1.0
D36G N43D	46 ± 9.6	1.7 ± 0.3
HIV-2		
EHO[b]	37 ± 10	1.5 ± 0.5

[a] EC_{50} was determined as the concentration that blocked HIV-1 replication by 50% in MAGI assay. [b] The antiviral activity (EC_{50}) of peptide **11** against EHO strain was 2.1 ± 0.7 nM.

Interestingly, peptide **2** showed potent antiviral activity even against an HIV-2 EHO strain [EC_{50} = 1.5 nM], which is as potent as peptide **11** having a congenerous sequence derived from the EHO strain [EC_{50} = 2.1 nM].[23] This is in contrast to the previous report on the reduced activity of **1** against the EHO strain [EC_{50} = 37 nM].[24] The potent bioactivity of **2** can be rationalized by the minimal difference of the interface residues between HIV-1 and -2 and the stabilized α-helix structure. Among 19 different residues between the sequences of NL4-3 and EHO strains, 13 residues are located at the solvent accessible sites (b, c, f, and g positions). Although the other six residues are possibly involved in the direct interaction, the potential reduced interactions derived from the mismatch could be recovered by introduction of X-EE-XX-KK motifs.

In conclusion, remodeling of **1** to yield the preordered α-helical structure of **2** led to improved affinity with NHR and increased antiviral activity even against enfuvirtide-resistant HIV-1 and HIV-2 strains. This approach also helped to clarify the potential minimal interface of **1** with viral gp41. Peptide **2** could be a useful chemical tool to understand the membrane fusion process of HIV-1 and the detailed action mechanism of enfuvirtide.

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Supporting Information Available: Experimental details for peptide preparation, CD spectra measurements, and bioassays and MS and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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