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Suitable fusion of N-terminal heptad repeats to achieve covalently stabilized potent N-peptide inhibitors of HIV-1 infection

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ABSTRACT

N-terminal heptad repeat (NHR)-derived peptide (N-peptide) fusion inhibitors, which are derived from human immunodeficiency virus (HIV) envelope glycoprotein 41 (gp41), are limited by aggregation and unstable trimer conformation. However, they could function as potent inhibitors of viral infection by forming a coiled-coil structure covalently stabilized by interchain disulfide bonds. We previously synthesized N-peptides with potent anti-HIV-1 activity and high stability by coiled-coil fusion and covalent stabilization. Here, we attempted to study the effects of NHRs of chimeric N-peptides by fusing de novo coiled-coil isopeptide bridge-tethered T21 peptides of different NHR lengths. Peptides $(T21N23)_3$ and $(T21N36)_3$ was a more potent HIV-1 fusion inhibitor than $(T21N17)_3$. The site of isopeptide bond formation was precisely controlled and had little influence on N-peptide properties. The N-peptide $(T21N36)_3$, which had a similar conformation as the NHR trimer and interacted well with the C34 peptide, may be useful for screening other C-peptides and small-molecule fusion inhibitors, and for studying the interactions between the NHR trimer and C-terminal heptad repeats.

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein 120 (gp120)/gp41 complex is the key mediator in the fusion of cell and viral membranes.[1,2] Specifically, the binding of gp120 to its cellular receptor and co-receptors[3,4] triggers a series of conformational changes in gp41[5] and simulates the formation of a six-helical bindle (6HB) between a central parallel trimeric coiled coil of N-terminal heptad repeats (NHRs) and C-terminal heptad repeats (CHRs), leading to the fusion of viral and targeting cellular membranes.[6,7] NHR-derived peptides (N-peptides) and CHR-derived peptides (C-peptides) can be potent fusion inhibitors by interrupting 6HB formation.[8] C-peptides inhibit HIV-1 infection by interacting with grooves of the NHR trimer,[9,10] and are active at low nanomolar concentrations. T20 (enfuvitide/Fuzeon) was approved by the U.S. Federal Drug Administration in 2003 as the first HIV-1 fusion inhibitor for the treatment of HIV/AIDS patients who fail to respond to current antiretroviral drugs.[11] However, the appearance of resistant HIV-1 strains and the short half-life of T20 in vivo[12,13] make it necessary to develop new fusion inhibitors to overcome these drawbacks and achieve proteolytic stability.[14,15]

Even though N-peptides can also inhibit HIV-1 entry, their activities are lower than those of C-peptides because of their unstable trimer conformation and tendency to aggregate in physiological conditions.[16] Thus, efforts have been made to improve N-peptide activity.[17,18] Stable trimer conformation is necessary for N-peptide inhibitors.[19,20] Attaching a soluble coiled-coil trimer to the NHR motif and covalent stabilization have also proven to be effective in increasing N-peptide activity, and as such, are garnering an increasing amount of attention.[21,22] The chimeric N-peptides IZN17[23] and N28Fd[24] and covalent trimers CCIZN17,[25] CCN28Fd,[26] and (HR3N23)3[27] reportedly have good anti-HIV-1 activity. In chimeric N-peptides, fusing coiled-coil trimers can stabilize the conformation and improve activity. However, it is unknown if lengthening the NHR sequence enhances the antiviral activity of the fused chimeric N-peptide. To address this question, we designed T21N17, T21N23, T21N36, (T21N17)3, (T21N23)3, and (T21N36)3 peptides by fusing coiled-coil T21 with N17, and N23, N36 followed by covalent stabilization. The coiled-coil T21 sequence was designed from de novo coiled-coil peptides containing three heptad repeats (3HR).[27] N17, N23, and N36 peptides were derived from the

Abbreviations: NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; 6HB, six helix bundle; DIEA, diisopropylethylamine; DMF, N,N-dimethylformamide; HBTU, O-benzotriazol-1-yl-N,N,N',N'-tetramethyl-uronium hexafluorophosphate; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry

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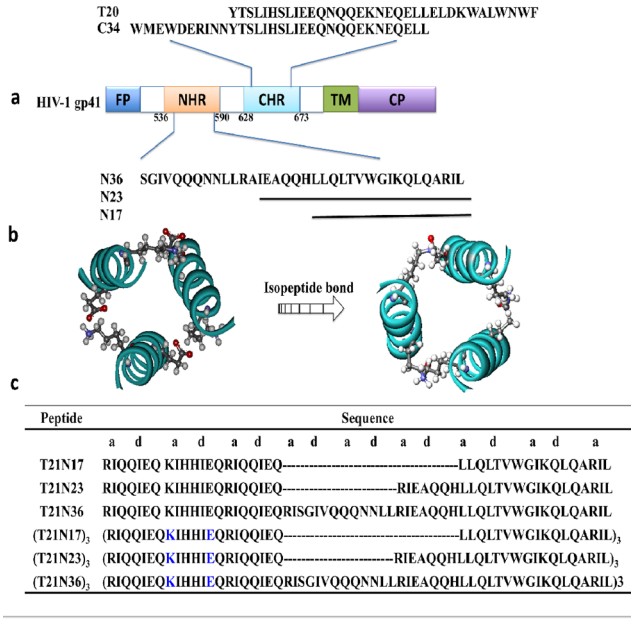

Fig. 1. (a) Schematic model of HIV-1 gp41 structure. (b) Schematic representation of isopeptide bond formation in the chimeric N-peptide trimers. (c) Peptide sequences of designed N-peptides. The isopeptide bond was formed by Glu-Lys and is shown in blue.

NHR of HIV gp41, and N17 was the pocket domain (Fig. 1a). These peptides had markedly increased antiviral potency against the corresponding NHR motifs themselves. The results showed that isopeptide stabilization (Fig. 1b) improved N-peptide structural stability and antiviral activity. The covalently stabilized peptide fused to suitable lengths of NHR motifs had stability against denaturation and higher antiviral activity. The N-peptide (T21N23)3 and (T21N36)3 inhibited HIV-1 entry with an IC50 of 6.9 nM and 13.5 nM in the cell-cell fusion assay.

2. Results and discussion

2.1. Peptide design

Previously, we designed an α-helical coiled-coil domain (3HR)3 to stabilize NHR motifs conformation and avoid their aggregation, and a Trp residue at the 3HR N-terminal was introduced to quantitate the peptide. Because the NHR pocket domain N17 contained a Trp residue, the truncated T21 coiled-coil trimer (sequence of Ac-RIQQIEQKIHHIEQRIQQIEQ-NH2) was fused to the NHR as the trimer structure stabilizer in this context. We designed the chimeric N-peptides T21N17, T21N23, T21N36 and (T21N17)3, (T21N23)3, (T21N36)3 by fusing the T21 coiled-coil trimer with the NHR motifs N17, N23, N36 and covalent stabilization. The N-peptides (T21N17)3, (T21N23)3, and (T21N36)3 were synthesized by modification of the corresponding thioester peptide (Fig. S1) and isopeptide bond cross-linking (Fig. 1c).

2.2. Isopeptide bond formation in the chimeric N-peptides

The designed N-peptide had a trimer helical structure that was determined by sedimentation velocity analysis (SVA, Fig. S2a). Then an acyl-transfer reaction was introduced to synthesize the covalently stabilized N-peptides between the helical trimer. On the basement N-peptide trimer structure, the Glu-13 side chain at the e position was benzyl thioester-modified (SBn) by a Glu(OAll) residue introduced into the standard solid-phase Fmoc synthesis and chemical modifications. Thus, the isopeptide bond-stabilized peptides were generated by

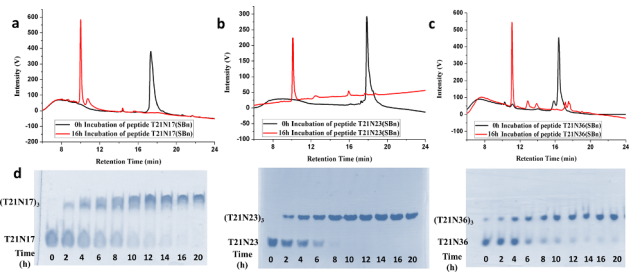

Fig. 2. (a) RP-HPLC was used to monitor the acyl transfer reaction of T21N17(SBn) at 0 and 16 h. RP-HPLC was used to monitor the acyl transfer reaction of T21N23(SBn) (b) and T21N36(SBn) (c) at 0 and 16 h. All of the acyl transfer products were verified by MALDI-TOF-MS. (d) SDS-PAGE was used to monitor the acyl transfer reaction at times 0–20 h.

thioester-modified peptide intermediates in a base environment (0.5 M phosphate-buffered saline [PBS], pH = 7.2). The reaction was monitored by reverse-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). In HPLC, the thioester-modified precursors displayed a rapid acyl-transfer rate, and little side product was formed. The molecular weight of the cross-linked peptide was determined by MALDI-TOF-MS, consistent with the theoretical mass values of the acyl-transfer product, indicating that an isopeptide bond was formed in the chimeric N-peptides. In SDS-PAGE (Fig. 2d), the upper bands were the acyl-transfer products and the lower bands were the thioester precursors. With reaction processing, the amount of product increased and the amount of precusors decreased. The results showed that the site of isopeptide bond formation was precisely controlled and could be extended to other helical coiled-coil proteins of similar structure.

2.3. Isopeptide bond stabilization improves the resistance of N-peptides to thermal denaturation

Circular dichroism (CD) spectroscopy was used to assess the natural and conformational stability of the covalently stabilized N-peptides (Table 1). CD data suggested that all of the N-peptides had an α-helix structure, with the amount of α-helicity ranging from 78% to 100%. In the thermal denaturation assay (Fig. S3), the covalently stabilized N-peptide had high thermal stability and no cooperative melting transition point (Tm) at temperatures up to 90 °C in 10 μM of a 2 M guanidine hydrochloride (Gdn·HCl) solution, whereas the chimeric N-peptides T21N17, T21N23, and T21N36 had Tm values of 72 °C, 74 °C, and 82 °C, respectively.

2.4. Covalently stabilized N-peptide exhibits highly potent anti-HIV-1 activity

We determined the antiviral activity of all of the N-peptides using

Table 1
Physicochemical properties of α-helicity, stability, and anti-HIV-1 activity were determined in the N-peptides.

Peptide	θ(deg·cm^2·dmol^{-1})	Helicity[a] (%)	Tm/(°C)	IC50[b] (nM)
T21N17	−25929	78	72	251 ± 41.1
T21N23	−32253	98	74	38.9 ± 35.8
T21N36	−28543	87	82	81.8 ± 5.69
$(T21N17)_3$	−35957	100	> 90	20.6 ± 4.37
$(T21N23)_3$	−32153	97	> 90	6.90 ± 1.29
$(T21N36)_3$	−30247	92	> 90	13.5 ± 7.65
N36				> 5000
T20				2.53 ± 0.65

[a] 100% helicity occurred at 33,000 deg·cm^2·dmol^{-1}.
[b] Median inhibitory concentration in 2 M Gdn·HCl.

the HIV-1 cell-cell fusion assay (Table 1). In the assay, the covalently stabilized N-peptides had more potent anti-HIV-1 activity than the corresponding chimeric N-peptides. (T21N17)3, (T21N23)3, and (T21N36)3 had IC50 values of 20.6, 6.9, and 13.5 nM, respectively. These results indicated that covalent stabilization improved the antiviral activity. Among the N-peptides, (T21N23)3 and (T21N36)3 had higher antiviral activity than (T21N17)3, indicating that lengthening the NHR motif improved N-peptide activity. (T21N36)3 had lower activity than (T21N23)3, possibly because the sequence was too long leading to a decreased its solubility.

2.5. Site of isopeptide bond formation has little influence on the N-peptide structure and anti-HIV-1 activity

To determine if the site of isopeptide bond formation affected the properties of the N-peptides, we designed (T21N17-n)3 and (T21N17-c)3 peptides, in which the site of bond formation was transferred to the N- and C-termini for seven repeats (residues Glu6 and Glu20; Tables S1, S2). The α-helicity and anti-HIV-1 of (T21N17-n)3 and (T21N17-c)3 was 92% and 24.6 nM and 96% and 16.7 nM, respectively, similar to that of (T21N17)3 (100%, 20.3 nM). These results indicated that transferring the position of the isopeptide bond had little influence on N-peptide structure and anti-HIV-1 activity.

2.6. Covalently stabilized N-peptide exhibits higher binding with CHR ligand C34

Studies have shown that N-peptides exert antiviral activity by interacting with the CHR of gp41 to prevent HIV-1 6HB formation.[19,28] Thus, CD spectroscopy was performed to investigate the interaction between the N-peptides and CHR ligand C34 (Fig. 3). When comparing the CD spectra of the N-peptides and C34 mixture and the mathematical sum of individual N-peptides and C34 spectra at a minimum intensity of 222 nm, we found that the CD intensities of covalently stabilized N-peptides and C34 mixture (specN-peptide/C34) were higher than the sum of individual peptides (specN-peptide + specC34), indicating that covalently stabilized N-peptides interacted with C34. This interaction led to stabilization of the α-helical conformation. Similar results were observed with native PAGE. In Fig. 3d, the C34 band can be seen in the lower position of the gel, and the N36/C34 mixture band (6HB complexed with N36/C34) migrated slowly and is in the upper position of the gel. A similar band was observed for the (T21N23)/C34, (T21N23)3/C34, (T21N36)/C34, and (T21N36)3/C34 complexes. Even though the (T21N17)3/C34 complex band was not clearly seen, the C34 band intensity was decreased in the same gel, indicating that the (T21N17)3/C34 complex formed, but did not have as stable a structure as N36/C34, constituent with the CD spectroscopy results.

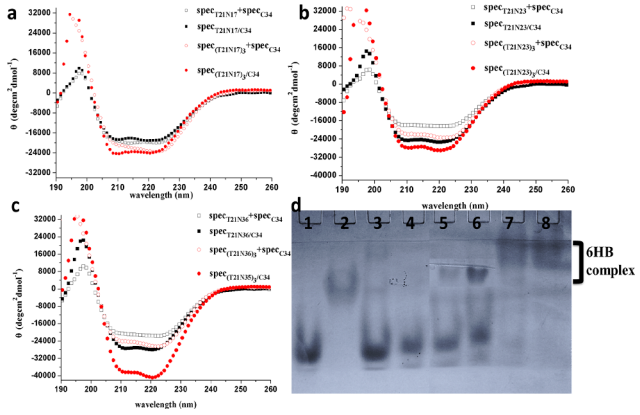

Fig. 3. CD spectra and N-PAGE of the N-peptides and C34 mixture. (a) T21N17 and (T21N17)3, (b) T21N23 and (T21N23)3, (c) T21N36 and (T21N36)3, (d) lane 1: C34, lane 2: N36/C34, lane 3: T21N17/C34, lane 4: (T21N17)3/C34, lane 5: T21N23/C34, lane 6: (T21N23)3/C34, lane 7: T21N36/C34, lane 8: (T21N36)3/C34.

3. Conclusions

In conclusion, the N-peptides were designed and synthesized by fusion of the de novo coiled-coil motif with NHRs and covalent stabilization. These N-peptides had good α-helical structure, conformation stability, and anti-HIV-1 activity. In this approach, suitably lengthening the NHR regions improved the anti-HIV-1 activity in the covalently stabilized N-peptides. The results also suggested that covalent stabilization could be extended to other proteins with coiled-coil motifs and isopeptide bond formation. Furthermore, the synthesized covalent trimer (T21N36)3 had a structure that was similar to the NHR trimer; thus, it may be useful for the high-throughput screening of C-peptides and small-molecule inhibitors that target NHRs, and for studying the interaction between NHRs and CHRs.

4. Materials and methods

4.1. Peptides synthesis

Peptides were synthesized by using the CS-Bio 136 automated peptide synthesizer (CSBio Co., Menlo Park, CA, USA) and a standard solid-phase Fmoc chemistry protocol. Synthesis was performed on the Fmoc-protected Rink amide resin, using Fmoc-protected amino acids. Coupling was achieved by using O-benzotriazol-1-yl-N,N,N′,N′-tetramethyl-uronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIEA) as a base activator mixture in N,N-dimethylformamide (DMF) solution. The Fmoc protective group was removed using 20% piperidine/DMF, and the resin was cleaved using Reagent K.

4.2. Synthesis of thioester-modified peptides

Peptides were first synthesized by solid-phase peptide synthesis using a standard Fmoc chemistry procedure, but the natural acid was replaced by Fmoc-L-glutamic acid O-allyl ester [Fmoc-Glu(OAll)–OH] at the thioester-modified site. After the peptide was synthesized on the resin, the O-allyl group was deprotected manually using the catalytic reagent palladium (0) (tetrakis(triphenylphosphine)palladium/Pd (PPh3)4, 1 equivalent) with 5,5-dimethyl-1,3-cyclohexanedione (10 equivalents) as a scavenger in the DCM/THF (1:1) solution. Then the resin was washed five times with 0.5% DIEA in DMF and five times with 0.5% sodium diethyldithiocarbamate in DMF. Next, the resin was treated twice with five equivalents of 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride and five equivalents of benzyl mercaptan for 6 h. Finally, the resin was cleaved using Reagent K.

4.3. Circular dichroism (CD) spectroscopy analysis

The secondary structure and melting temperature(Tm) of the engineered peptides were analyzed using a CD spectrometer (MOS-450; BioLogic Science Instruments, Seyssinet-Pariset, France) at a 4.0 nm bandwidth, 0.1 nm resolution, 0.1 cm path length, 4.0 s response time, and 50 nm/min scanning speed. The N-peptides were dissolved in ddH2O and diluted to 10 μM in PBS (pH = 7.2).

4.4. HPLC analysis of covalent bond formation in the N-peptide trimer

The thioester-modified N-peptide (300 μM) was dissolved in 1 mL mixed solvent (40% PBS, 60% H2O), and incubated at 30 °C. A volume of 60 μL samples were collected at different time points followed by the addition of 5 μL 30% trifluoroacetic acid to quench the reaction and storage at −20 °C. Samples were analyzed by HPLC.

4.5. Sedimentation velocity analysis (SVA)

All measurements were performed on 4 Proteome lab TMXL-A/XL-l analytical ultracentrifuge at 25 °C as previously described.[22] In brief, cells were centrifuged in the AN-60 Ti Analytical Rotor. The N-peptides and C34 peptide were dissolved in ddH2O and PBS to a concentration of ~1 mg/mL. Then equimolar concentrations of individual N-peptides and C34 mixtures were prepared in PBS to a concentration of ~120 μM and incubated for 30 min at 37 °C. All samples were centrifuged at 3000 rpm for 10 min. Data were collected at 60,000 rpm at a wavelength of 280 nm. The sedimentation coefficient distribution c(s), and molecular mass distribution c(M), were calculated using the SEDFIT program.

4.6. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted to detect the covalent complex formed in the N-peptides using 12% polyacrylamide gels and the BayGene Mini Cell (BioRad, Hercules, CA, USA). The cathode buffer was 0.1 M tricine, 0.1 M Tris, and 1% SDS; and the anode was 0.2 M Tris. The samples were mixed with SDS-PAGE sample buffer at a ratio of 1:1 by boiling in a water bath for 10 min, followed by loading onto the gels (20 μL/well). Gel electrophoresis was performed at 60 V for 40 min and then 100 V for 1.5 h at room temperature. Then the gel was stained with Bio-Safe Coomassie Stain (Bio-Rad, Hercules, CA, USA).

4.7. Native polyacrylamide gel electrophoresis (N-PAGE)

Native PAGE analysis was performed using 12% polyacrylamide gels and the BayGene Mini Cell. N-peptides in water were mixed with C34 in PBS and incubated for 30 min at 37 °C. The samples were mixed 1:1 with sample buffer and loaded onto the gels. Gel electrophoresis was conducted at a constant voltage of 120 V at room temperature for 3 h, after which the gel was stained with Bio-Safe Coomassie Stain (Bio-Rad).

4.8. Cell-cell fusion assay

Cell-cell fusion assays were conducted as previously described.[29,30] HL2/3 cells that stably express HIV Gag, Env, Tat, Rev, and Nef proteins and TZM-bl cells that stably express high levels cluster of differentiation 4 and C-C chemokine receptor type 5 were obtained from the NIH AIDS Research and Reference Reagent Program. TZM-bl cells were cultured for 24 h in a 96-well plate at 37 °C. Inhibitor samples were added and HL2/3 cells followed by the addition of new culture medium; the co-cultures were incubated for 6 h at 37 °C. Cell-cell fusion was determined by measuring luciferase activity using the Luciferase Assay System (Promega, Madison, WI, USA) and the SpectraMax M5 plate reader (Molecular Devices, Downingtown, PA, USA).

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Notes: The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.115214.

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