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HIV-1 variants with a single-point mutation in the gp41 pocket region exhibiting different susceptibility to HIV fusion inhibitors with pocket- or membrane-binding domain

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ABSTRACT

Enfuvirtide (T20), the first FDA-approved peptide HIV fusion/entry inhibitor derived from the HIV-1 gp41 C-terminal heptad-repeat (CHR) domain, is believed to share a target with C34, another well-characterized CHR-peptide, by interacting with the gp41 N-terminal heptad-repeat (NHR) to form six-helix bundle core. However, our previous studies showed that T20 mainly interacts with the N-terminal region of the NHR (N-NHR) and lipid membranes, while C34 mainly binds to the NHR C-terminal pocket region. But so far, no one has shown that C34 can induce drug-resistance mutation in the gp41 pocket region. In this study, we constructed pseudoviruses in which the Ala at the position of 67 in the gp41 pocket region was substituted with Asp, Gly or Ser, respectively, and found that these mutations rendered the viruses highly resistant to C34, but sensitive to T20. The NHR-peptide N36 with mutations of A67 exhibited reduced anti-HIV-1 activity and decreased α-helicity. The stability of six-helix bundle formed by C34 and N36 with A67 mutations was significantly lower than that formed by C34 and N36 with wild-type sequence. The combination of C34 and T20 resulted in potent synergistic anti-HIV-1 effect against the viruses with mutations in either N- or C-terminal region in NHR. These results suggest that C34 with a pocket-binding domain and T20 containing the N-NHR- and membrane-binding domains inhibit HIV-1 fusion by interacting with different target sites and the combinatorial use of C34 and T20 is expected to be effective against HIV-1 variants resistant to HIV fusion inhibitors.

1. Introduction

The fusion event of the human immunodeficiency virus type 1 (HIV-1) with its target cells is mediated by two noncovalently linked glycoprotein subunits, gp120 and gp41 [1]. Initially, gp120 binds to the cellular receptor CD4 and a coreceptor CCR5 or CXCR4, triggering a series of conformation rearrangements in gp41. Then, gp41 fusion peptide (FP) exposes and inserts itself into the target cell membrane [2–4]. Conformational changes subsequently occur, bringing the gp41 N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR) domains (Fig. 1A) together to form a thermostable six-helix bundle (6-HB) core structure [5–7], which then brings the host and viral membranes into close proximity for membrane fusion [8,9].

The peptides derived from the CHR domain, such as SJ-2176 (residues 119–148) [10,11], DP-178 (also known as T20; residues 127–162) [12,13], and C34 (residues 117–150) [6,14], exhibit potent HIV-1 fusion inhibitory activity. Some of the peptides derived from the NHR domain, such as DP-107 (also known as T21; residues 42–79) [15] and N36 (residues 35–70) [6,14], also show anti-HIV-1 activity. C34 is the best-characterized CHR-peptide because the crystal structure of the complex of C34 and N36 was solved [6]. It was shown that C34 binds in an antiparallel manner into hydrophobic grooves on the outer surface of the internal trimeric NHR coiled-coil formed by N36. A deep hydrophobic pocket is formed by residues 54–70 of the C-terminal region of NHR domain in each of the grooves of NHR-trimer. The residues in this pocket are believed to be crucial

Abbreviations: NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; 6-HB, six-helix bundle; Env, envelope protein; HBD, heptad-repeat binding domain; PBD, pocket-binding domain; PFD, pocket-forming domain; MBD, membrane-binding domain; CD, Circular Dichroism; N-PAGE, native polyacrylamide gel electrophoresis; $TCID_{50}$, 50% tissue culture infective dose; IC_{50}, concentration causing 50% inhibition

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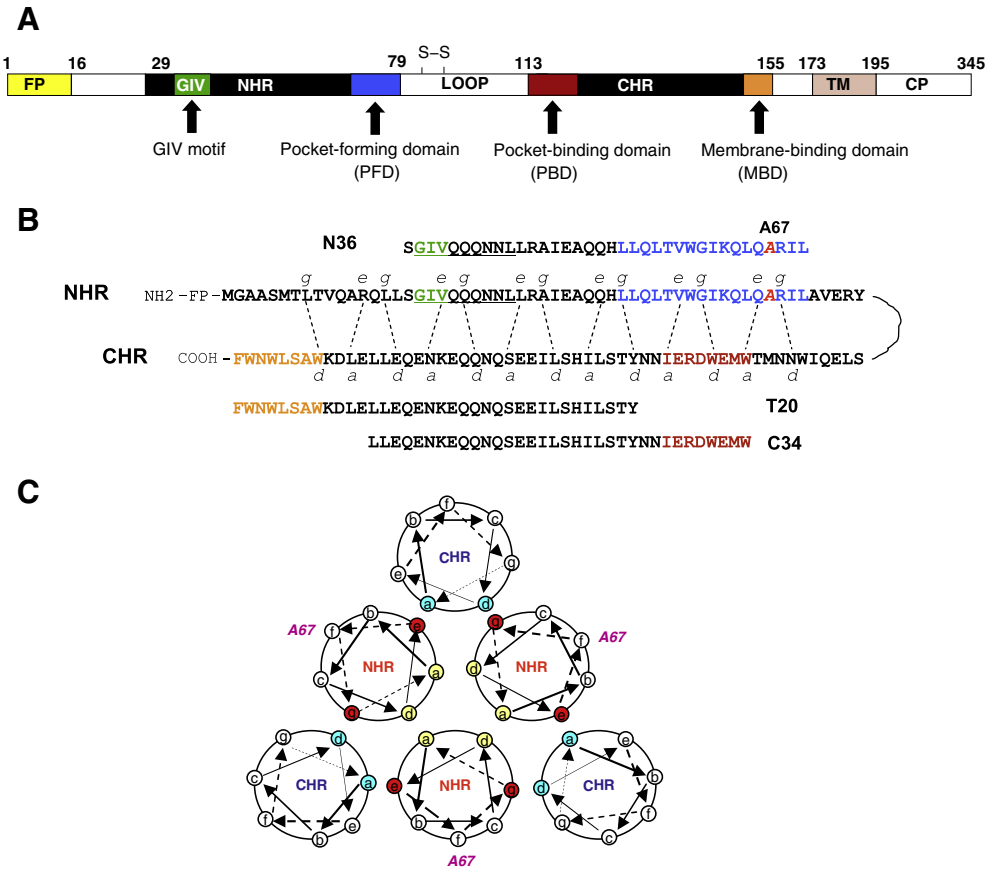

Fig. 1. Functional domains and hydrophobic pocket of HIV-1 gp41. (A) Schematic representation of gp41 functional domains and corresponding peptide sequences. The residue number corresponds to HIV-1 HXB2 gp160 sequence. FP, fusion peptide; TM, transmembrane domain; CP, cytoplasmic domain; PBD, pocket-binding domain; PFD, pocket-forming domain; MPD, membrane-binding domain. (B) Interaction between the NHR and CHR. The letters "*a*–*g*" indicate the positions of the corresponding residues in the helical wheel of the gp41 NHR domain. Residue A67 is located at the *f* site in the helical wheel of the gp41 NHR domain, which does not involve direct interaction with the key residues in NHR for forming N-trimer or those in CHR for forming 6-HB. The sequences of N36, T20 and C34 are also shown. (C) Model of 6-HB formed by the N- and C-helices. The 6-HB is formed through the interaction of residues located at the *e* and *g* positions (in *red*) in the N-helices and those at *a* and *d* positions (in *blue*) in the C-helices. Residue A67 located at the *f* site is also shown.

for 6-HB stability and viral infectivity [16–19]. Similarly, the residues in the pocket-binding domain (PBD; residues 117–124) in C34 peptide and other PBD-containing CHR-peptides are critical for the formation of 6-HB with the viral gp41 NHR domain and for their anti-HIV-1 activity [18–22].

T20 (generic name: enfuvirtide; brand name: Fuzeon) is the first U.S. FDA-approved HIV fusion/entry inhibitor for clinical use [23,24]. Since both T20 and C34 peptides have an overlapping sequence in CHR domain,viruses with mutations in the GIV motif (residues 36–44) in the N-terminal region of the NHR domain are rendered highly resistant to T20 [25–30] and partially resistant to C34 [31]. It has been widely believed that T20 and C34 share a common target, i.e., inhibition of HIV fusion with the target cell membrane by binding to the viral NHR domain to block the fusion core formation. However, our previous studies have suggested that T20 and C34 may have different target sites because they contain different functional domains [21,22]. C34 mainly interacts through its PBD with essentially the pocket region in the C-terminal region of NHR domain, to form a stable 6-HB and block viral fusion core formation. However, T20, which lacks the PBD, mainly binds to the N-terminal region of the NHR domain (N-NHR), where the GIV motif is located, and interacts with the cell membrane via its membrane-binding domain (MBD) or tryptophan-rich domain (residues 155–162) [20–22,32,33]. Shai and colleagues have demonstrated that T20's MBD (WNWF) is critical for its antiviral activity since replacement of WNWF in T20 with ANAA results in loss of its anti-HIV-1 activity, while addition of an ocytl group to the C-terminus of the T20-ANAA mutant rescues this inactive mutant to an anti-HIV-1 potency similar to that of the wild-type T20 [32]. However, our hypothesis is not verified by mutation studies since it has not, thus far, been shown that the CHR-peptides containing the PBD could induce drug-resistant mutations in the gp41 pocket region.

In this study, we aimed to circumvent this obstacle by arbitrarily introducing mutations into the pocket region in gp41 of pseudoviruses and comparing the susceptibility of the mutant viruses with that of the wild-type virus to C34 and T20. We found that the substitutions of Ala at the position of 67 in the gp41 NHR C-terminal pocket region with Asp, Gly or Ser (A67D/G/S) rendered the viruses resistant to C34, but sensitive to T20. We also demonstrated that the combination of C34 and T20 exhibited strong synergistic antiviral effect against HIV-1 strain IIIB and those strains resistant to T20 and/or C34. These findings confirm that C34 and T20 inhibited viral fusion by interacting with different target sites. The combinatorial use of T20 with C34 and other CHR-peptides containing PBD resulted in synergistic anti-HIV-1 activity, thus suggesting a potential regimen for treating patients infected by HIV-1 isolates with resistance to HIV entry inhibitors.

2. Materials and methods

2.1. Materials

TZM-bl and MT-2 cell lines, HIV-1 strain IIIB, NL4-3 and its mutants, and the plasmid encoding HIV-1 HXB2-Env (pHXB2-Env) were

acquired from the NIH AIDS Research and Reference Reagent Program. Peptides N36 and its mutants (N36-A67D, N36-A67G and N36-A67S), C34 and T20 were synthesized by a standard solid-phase FMOC method using an Applied Biosystems model 433A peptide synthesizer. The N- and C-termini of these peptides were acetylated and amidated, respectively. The peptides were purified to homogeneity (>95% purity) by high-performance liquid chromatography and verified by laser desorption mass spectrometry (PerSeptive Biosystems, Framingham, MA). The concentration of these peptides was decided by UV absorbance at 280 nm, according to the theoretically calculated molar extinction coefficients (ε) (280 nm) of 5500 and 1490 mol/l^{-1} cm^{-1} based on the number of tryptophan (Trp) residues and tyrosine (Tyr) residues (all peptides tested contain Trp and/or Tyr), respectively [34].

2.2. Generation of HIV-1 pseudoviruses

Pseudovirus with wild-type, or mutant, Env protein was produced and evaluated for infectivity as previously described [35,36]. Briefly, A67D, A67G and A67S mutations were introduced into the pHXB2-Env using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The plasmid encoding wild-type or mutant HXB2-Env and another plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.Luc.R-E-) were co-transfected into HEK293T cells by using FuGENE 6 reagents (Roche Applied Science). The supernatants were collected at 48 h post-transfection. The supernatants were centrifuged at 1000 ×*g* for 10 min, filtrated through a 0.45-μm filter, and then concentrated by centrifuging at 26,000 ×*g* for 2 h. The concentrations of these pseudovirion stocks were determined by enzyme-linked immunosorbent assay (ELISA) for p24 antigen (Coulter) and stored at 80 °C.

2.3. Single-cycle infection and drug inhibition assays

About 10^4 TZM-bl cells/well were plated in 96-well dishes in Dulbecco's Modified Eagle's Medium (DMEM). The dosage of pseudovirion stocks was fixed at approximately 250 ng of p24 per ml for single-cycle infection and 200 ng of p24 per ml for viral inhibition assay. The pseudovirus infected the cells in the presence or absence of peptide in a total volume of 100 μl with 10 μg of Polybrene (Sigma) per ml. After 16 h of incubation, the medium was changed with 100 μl of fresh DMEM, and the cultures were left to grow for an additional 48 h at 37 °C. Then the cells were harvested, washed with PBS and lysed using the lysis reagent from a luciferase kit (Promega, Madison, WI). The cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega), and tested immediately for the luciferase activity (relative light units: RLU) in an Ultra 384 luminometer (Tecan US) [37]. The relative infection activity was calculated using the following formula: $[(E-N)/(P-N)]\times 100\%$, where *E*, *P* and *N* represent the luciferase activity (RLU) of the experimental group (cells + peptide + virus), the positive control group (cells + PBS + virus), and the negative control group (cells + PBS + PBS), respectively.

2.4. CD spectroscopy

The secondary structure of N peptides and the mutants (or their complexes with C peptide) was determined by CD spectroscopy. All the peptides were dissolved in PBS to 10 μM (pH 7.2). An N-peptide (N36 or its mutants) was incubated with the C-peptide (C34) at 37 °C for 30 min before measurement. The separated N- and C-peptides were also measured. Circular dichroism (CD) spectra of these peptides and peptide mixtures were acquired on a Jasco spectropolarimeter Model J-715 (Jacobin, Japan) at room temperature using a 5.0 nm bandwidth, 0.1 nm resolution, 0.1 cm path length, 4.0 sec response time, and a 50 nm/min scanning speed. The spectra were corrected by subtraction of a blank corresponding to the solvent. The α-helicity was determined from the CD signal by dividing the mean residue ellipticity at 222 nm according to a previously used value of 33,000 degrees dmol^{-1}as 100% helix formation [38]. Thermal denaturation of the protein was inspected at 222 nm by monitoring a temperature gradient from 5 °C to 80 °C with a 2-degree interval, an equilibration time of 1.5 min, and an averaging time of 60s.

2.5. Native polyacrylamide gel electrophoresis (N-PAGE)

N-PAGE was used to detect the effect of the mutations on formation of 6-HB between the N-peptide and C-peptide as described previously [39]. Briefly, the mixture of an N-peptide (N36 or its mutants) and C-peptide C34 with different concentrations (60 μM, N36; 60 μM, C34) was incubated at 37 °C for 30 min and loaded onto Tris-glycine gels (18%; Invitrogen). Then gel electrophoresis was carried out at 125 V constant voltages at room temperature for 2 h. The gels were stained with Coomassie Blue and imaged with a FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA).

2.6. Measurement of HIV-1_{IIIB} infectivity

The inhibitory activity of peptide N36 or its mutants on infection by a laboratory-adapted HIV-1 strain (IIIB) was tested as previously described [40]. Briefly, 1×10^4/ml MT-2 cells were infected with HIV-1 isolates at 100 $TCID_{50}$ (50% tissue culture infective dose) in 200 μl RPMI 1640 medium in the presence or absence of the test peptides overnight. Then the culture supernatants were taken out, and fresh media were added. On the fourth day post-infection, 100 μl of culture supernatants were collected from each well, mixed with equal volumes of 5% Triton X-100, and analyzed for p24 antigen by ELISA as previously described [41].

2.7. Combination study

The assays for evaluating HIV-1 Env-mediated cell-cell infection and HIV-1 infection were used to test the synergistic antiviral effect of the T20/C34 combination as previously described [42]. T20 and C34 were measured individually, or in combination, at a fixed molar ratio for the greatest synergism over a range of serial dilutions. The results were analyzed as previously described [43,44]. The analysis was executed progressively by calculating IC50 (or IC75, IC90, and IC95) values based on the inhibition curves of single drug or two drugs tested in combination. Then, the combination index (CI) was determined by calculating the median effect equation with the CalcuSyn program (kindly provided by Dr. T. C. Chou at the Memorial Sloan-Kettering Cancer Center) to assess the synergistic effect of combinations. A CI of <1 indicates synergism (CI values are interpreted as follows: <0.1, very strong synergism; 0.1 to 0.3, strong synergism; 0.3 to 0.7, synergism; 0.7 to 0.85, moderate synergism; and 0.85 to 0.90, slight synergism). A CI of 1, or close to 1, indicates additive effects, and a CI of >1 indicates antagonism [43]. Dose reduction was obtained by dividing the IC50 value of a peptide tested alone by that of the same peptide tested in combination with other peptides.

3. Results

3.1. Generation of infectious pseudoviruses expressing HIV-1 Env with mutations of residue A67 in the pocket-forming region of the HIV-1 gp41 NHR domain

To generate infectious virions with mutations in the gp41 pocket region, we must avoid changing the residues at the *a, d, e,* and *g* sites in the helical wheel of the NHR coiled-coil domain since these residues are responsible for mediating the association among the NHR domains

to form internal NHR-trimer or the interaction between the NHR and CHR domains to form 6-HB core. These residues are highly conserved, and mutations of these residues may result in noninfectious virions [18,45,46]. In this study, we selected residue A67 for mutations since its locus is the *f* site in the helical wheel of the NHR domains (Fig. 1A and C). Since it is not located on the hydrophobic interface for interaction among NHRs or interaction with CHR, mutation of this residue would not significantly affect viral infectivity. We constructed three pseudoviruses with the substitutions of A67 in the gp41 pocket region with Asp, a negatively charged residue (A67D), or Gly, a residue of smaller size (A67G), and Ser, a residue with polarity (A67S). These point mutations were confirmed by sequencing the entire Env gene. Their infectivity was determined by a luciferase assay. As shown in Fig. 2, the infectivity of mutant virus A67G had no significant decrease, while the A67D and A67S mutants possessed essential infectivity of about 45% of the infectivity exhibited by the WT virus. This result suggests that mutations of A67 do not completely disrupt viral infectivity and that pseudoviruses with these mutations can be used to evaluate viral susceptibility to antiviral agents.

3.2. Pseudoviruses with mutations of A67 in the gp41 pocket region were resistant to C34, but relatively sensitive to T20

We investigated the possible susceptibility of the mutant viruses to the peptide HIV fusion inhibitors with or without pocket-binding sequence. As shown in Table 1 and Fig. 3, all the variants with substitutions at position 67 in the pocket region showed high resistance to C34 (ranging from 61- to 244-fold). However, while the viruses with A67G mutation showed no resistance to T20, those with A67S and A67D exhibited a low level of resistance to T20. These results indicate that the mutation in the C-terminal pocket region of the gp41 NHR domain results in increased resistance to C34 which contains the pocket-binding sequence, while the viruses with these mutations retain their sensitivity to T20, which lacks the pocket-binding sequence. We also compared the sensitivity of the virus with V38E/N42S mutations in the GIV motif in the N-terminal region of the gp41 NHR domain to T20 and C34. This virus was very resistant to T20 (about 105-fold) and moderately resistant to C34 (about 5-fold), results which are consistent with our previous reports [37].

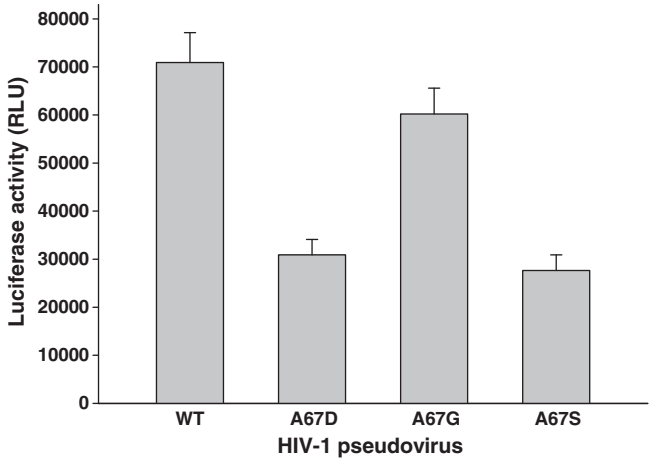

Fig. 2. Infectivity of HIV-1 pseudoviruses bearing wild-type or mutant HXB2 Env. Single-cycle infection of the different pseudoviruses bearing A67 mutations on TZM-bl cells was measured by a luciferase-based assay using luciferase kit from Promega. The luciferase activity (relative light units: RLU) was detected using an Ultra 384 luminometer from Tecan US. WT, wild type.

Table 1
The resistance profile of NL4-3 variants with mutations in the pocket-forming region of the HIV-1 gp41 NHR domain to the HIV-1 fusion inhibitors T20 and C34.

Virus	T20		C34	
	IC_{50} (nM)	Resistance (n-fold)	IC_{50} (nM)	Resistance (n-fold)
WT	17 ± 0.01	1.0	10 ± 0.01	1.0
V38E/N42S	1790 ± 100.2	105.3	54.13 ± 4.33	5.4
A67D	157 ± 8.5	9.2	1025 ± 6.33	102.5
A67G	21 ± 0.11	1.2	606 ± 20.1	60.6
A67S	58 ± 0.32	3.4	2435 ± 12.7	243.5

IC_{50} values are presented in nM. Results are derived from experiments performed in duplicate and are representative of at least three independent experiments.

3.3. Mutations of A67 in the gp41 pocket region resulted in decreased α-helicity and stability of 6-HB formed by N36 and C34

To understand the resistance mechanism of A67 mutants, we studied the biophysical properties of NHR-peptide N36 and its mutants using CD spectroscopy. As shown in Fig. 4 and Table 2, the isolated N36 peptide displayed low α-helicity (29.9%), which is consistent

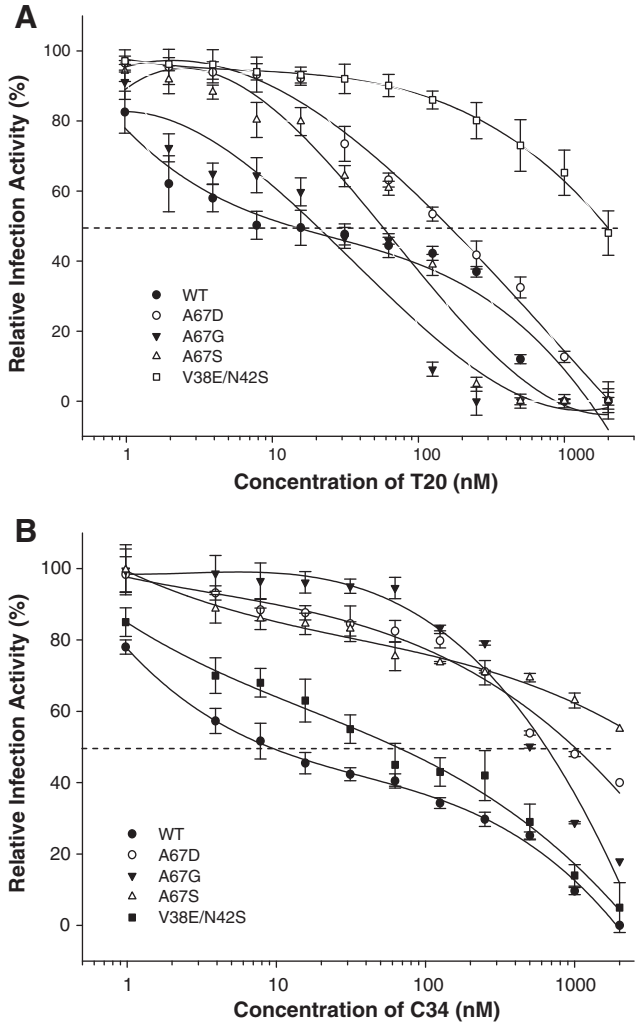

Fig. 3. Inhibitory activity of HIV-1 fusion inhibitors on infection of pseudoviruses expressing wild-type and mutated gp41. Single-cycle infection experiments using TZM-bl cells were performed to test the inhibitory activity of T20 (A) and C34 (B) on infection of the pseudoviruses with or without mutation. The relative infection activity of each pseudovirus in the presence of an inhibitory peptide was calculated based on their luciferase activity (RLU) in comparison with that of the virus in the absence of the inhibitory peptide. Error bars were standard deviation (SD) from at least three independent experiments.

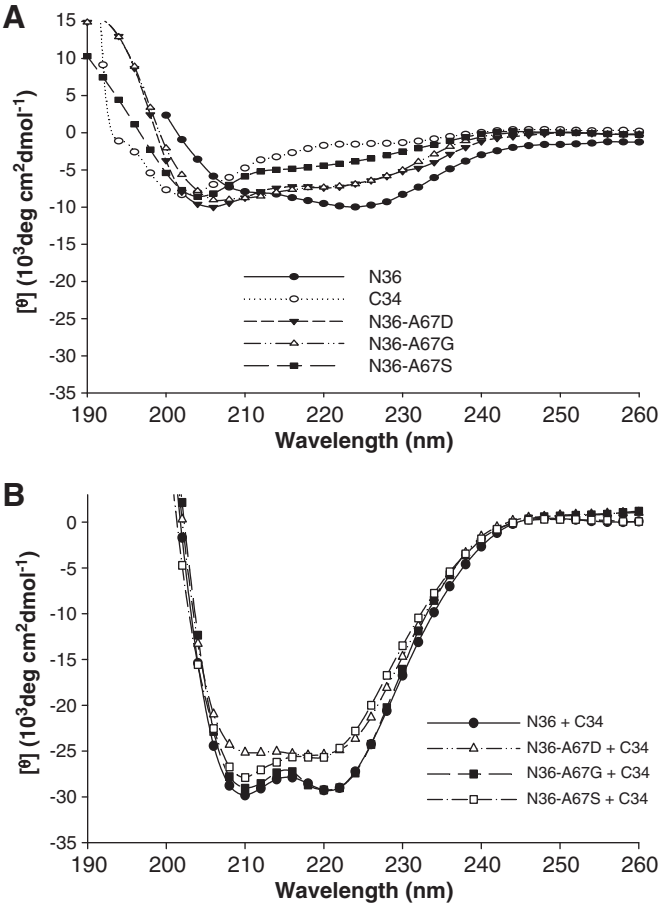

Fig. 4. CD spectra of separated N-peptides or the mixture of N-peptides and C-peptide. CD spectroscopy was used to determine the secondary structure of N peptides N36 and its mutants (A) and 6-HB formed by the mixture of C34 with N36 or its mutants (B). Both the N- and C-peptides were dissolved in PBS (pH 7.2) to 10 μM. The α-helicity was calculated from the CD signal as described in the Materials and Methods.

with reports in the literature [22], while the mutant peptides N36-A67D, N36-A67G, and N36-A67S displayed reduced α-helicity in the range of 12.6% to 22.0%. Then, we analyzed the secondary structures of the 6-HB formed by the interaction of C34 with N36 and its mutants. The mixture of C34 and N36 with WT sequence at equimolar concentration formed a typical helical conformation with high α-helicity, as shown by the obvious saddle-shaped negative peak in the wavelength of 200–230 nm in the CD spectrum. All A67 mutants could still interact with C34 to form 6-HBs, but their α-helical contents were significantly reduced. To determine the stability of the variant 6-HBs formed by N- and C-peptides, we performed thermal denaturation analysis by monitoring the CD signal at 222 nm over a temperature range. The melting curves of each protein and their thermal unfolding transition (Tm) values were shown in Table 2. The Tm of the 6-HB formed by C34 and the wild-type N36 was 66 °C, while the Tm values of those formed by C34 and the N36 mutants decreased to 53–62 °C. These results suggested that mutations of A67 in the gp41 pocket region can impair the ability of NHR domains to form α-helical coiled-coil structure, which is important for viral fusion.

Table 2
The α-helical content of N36 and its mutants, as well as their mixtures with C34.

N-peptide	[θ] 222 nm (×10³)	% Helicity	Tm (°C)
N36	−9.9	29.9	NA
N36 A67D	−7.2	22.0	NA
N36 A67G	−7.1	21.0	NA
N36 A67S	−4.1	12.6	NA
N36 + C34	−31.6	96	66
N36 A67D + C34	−24.8	75	53
N36 A67G + C34	−28.0	87	62
N36 A67S + C34	−24.6	74	58

The thermal stability of 6-HB formed by the mixture of N-peptide with C34 at 10 μM in PBS (pH 7.0) for CD measurement.

3.4. Substitutions of A67 might change the conformation of 6-HBs

To test if the mutations affect the configuration of the variant 6-HB formed by N- and C-peptides, we used N-PAGE to visualize the 6-HB formed by N36/C34 [22,39]. As shown in Fig. 5, the peptide N36 (lane 1) and its mutants exhibited no band because they carries net positive charges and may migrate off the gel [22]. C34 exhibited a band shown in the lower portion of the gel, which is consistent with a previous study [22]. When N36 and its mutants were mixed with C34 peptide, the bands corresponding to the variant 6-HBs appeared. However, all bands of 6-HB formed by the C34 and N36 mutants shifted downward in the gel. Since the locations of these 6-HBs in the gel are determined by several factors, such as net charge, shape, and molecular size, this result suggests that the configuration of the 6-HBs formed by C34 and N36 with A67 mutations may change.

3.5. Mutations of A67 in N36 peptide resulted in significant reduction of peptide-mediated anti-HIV-1 activity

The NHR peptide N36 possesses anti-HIV-1 activity since it can interact with the viral gp41 CHR domain and block viral gp41 6-HB core formation. To determine the potential effect of A67 mutations on the functionality of N36, the inhibitory activities of N36 and its mutants upon infection by HIV-1 IIIB were measured. As shown in Fig. 6, wild-type N36 potently inhibited viral infectivity with IC_{50} at about 1 μM, while the N36 mutants exhibited significantly reduced anti-HIV-1 activity with IC_{50} values ranging from 2 to 14 μM. These data were consistent with the α-helicity and Tm values of the mutant peptides as determined by CD spectroscopy (Table 2).

3.6. Combining T20 with C34 produced exceptionally potent synergism against HIV-1-mediated cell-cell fusion and infection by HIV-1_{IIIB} and resistant strains

Since T20 and C34 may adapt different mechanisms to inhibit HIV-1 infection, we investigated the potential cooperative effects of T20 with C34 on HIV-1-mediated cell-cell fusion (Table 3). Strikingly,

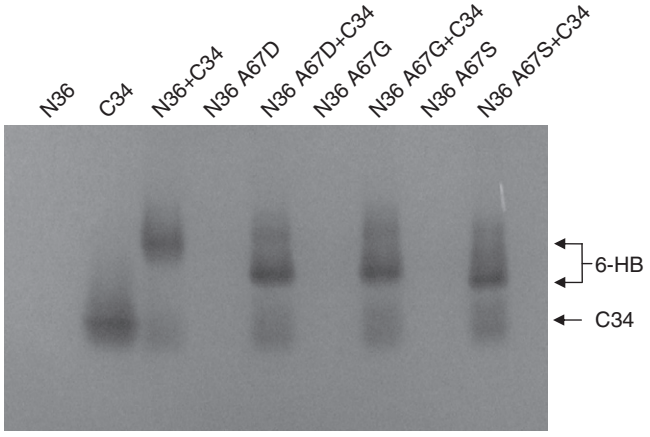

Fig. 5. Determination of the 6-HB formation between wild-type/mutant N36 and C34 by N-PAGE. The bands of the individual C-peptides and 6-HBs formed by the N- and C-peptides in the gel were revealed by Coomassie Blue staining. Final concentration of each peptide is 60 μM. N-peptide N36 and its mutants exhibited no band because they carried net positive charges and may migrate off the gel.

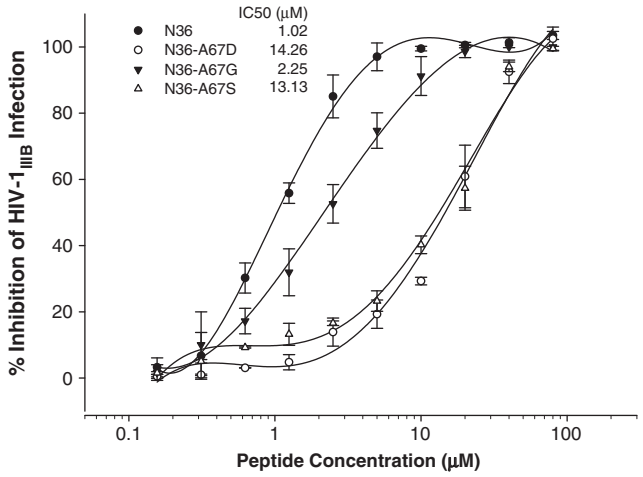

Fig. 6. Anti-HIV activity of wild-type and mutant N36 peptides. Infectivity of HIV-1_{IIIB} (100 $TCID_{50}$) on MT-2 cells in the presence or absence of tested peptides was measured by ELISA for p24 production. Each sample was tested in triplicate and the data were presented in mean ± SD. The IC_{50} (μM) values of the corresponding N-peptides were shown in the figure.

the combination of T20 with C34 caused noteworthy synergism, with a 400- to 600-fold dose reduction. We then tested the potential synergistic effect against infection by laboratory-adapted HIV-1_{IIIB} strain. We obtained a similar result, i.e., combining T20 and C34 resulted in a potent synergism with a CI of <0.01 (Table 3). We further determined whether the combination of T20 and C34 is also synergistic against HIV-1 strains resistant to T20 and/or C34. As shown in Fig. 7, the T20/C34 combination exhibited potent synergistic antiviral effect against both T20-resistant strain V38E/N42S and C34-resistant strain A67S, suggesting that T20 and C34 may be used in combination for treating patients infected by HIV-1 strains resistant to HIV fusion inhibitors targeting gp41.

4. Discussion

T20, which is derived from the C-terminal sequence of the HIV-1 gp41 CHR domain, is a potent HIV-1 fusion inhibitor [12,13,24,47,48]. In 2003, it was licensed by the U.S. FDA as the first member of a new class of anti-HIV drugs known as HIV fusion/entry inhibitors for the treatment of HIV-infected patients who fail to respond to the current antiretroviral therapeutics [24]. CD analysis showed that T20 could disrupt the stable α-helical structure of the NHR-peptide DP-107 and that the inhibitory activity of T20 correlated with its ability to interact with the NHR domain of gp41 [12,13,49]. These observations led to a broad belief that T20 inhibits HIV-1 fusion and entry by blocking viral gp41 6-HB core formation, much like C34 which is derived from the N-terminal region of the gp41 CHR domain and partially overlaps the sequence of T20.

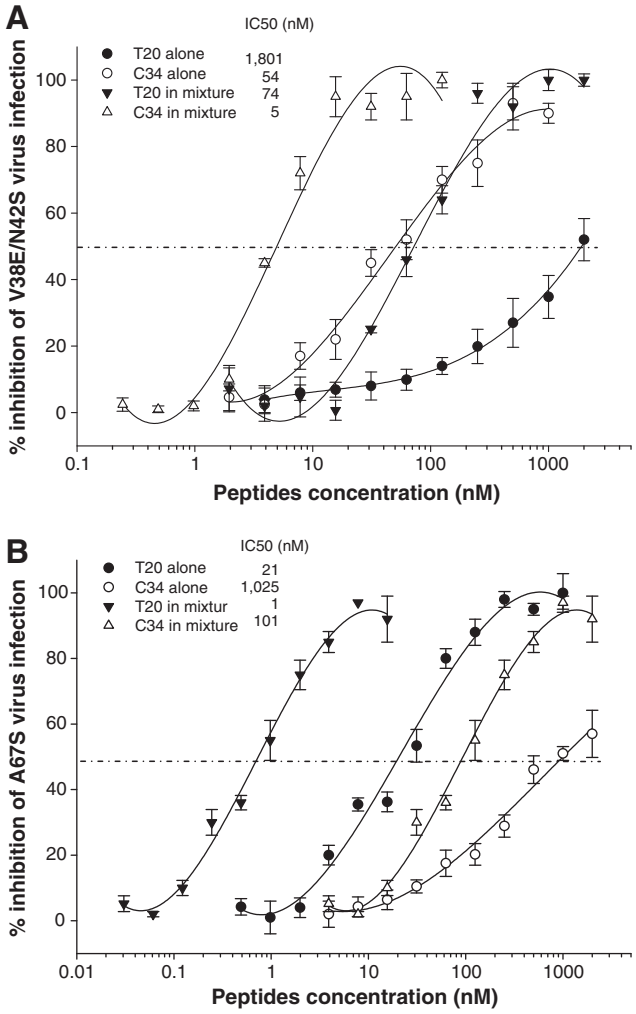

Fig. 7. Synergistic effect of combinations of T20 with C34 on inhibition of pseudoviruses with mutation V38E/N42S (A) or A67G mutation (B). The potential synergistic antiviral effect of the T20/C34 combination on inhibition of HIV-1 Env-mediated cell-cell infection and HIV-1 infection were tested as previously described [42]. The IC_{50} (nM) values of T20 alone, C34 alone, T20 in the mixture or C34 in the mixture for inhibiting infection by V38E/N42S mutant or A67G mutant, respectively, were shown in the figure. Each sample was tested in triplicate and the data were presented in mean ± SD. The experiment was repeated twice, and a representative set of data is shown.

Table 3
Combination index and dose reduction values for inhibition of HIV-1-mediated cell-cell fusion and HIV-1 IIIB infection by combinations of T20 and C34[a].

% Inhibition	CI	T20			C34		
		Concentration (nM)		Dose reduction	Concentration (nM)		Dose reduction
		Alone	Mixed		Alone	Mixed	
Inhibition of HIV-1-mediated cell-cell fusion							
50	0.004	10.490	0.023	456.09	2.074	0.003	691.33
75	0.004	13.806	0.029	476.07	2.614	0.004	653.50
90	0.003	18.171	0.038	478.18	3.295	0.005	659.00
95	0.003	21.905	0.045	486.78	3.856	0.006	642.67
Inhibition of HIV-1 $_{IIIB}$ infection							
50	0.040	19.799	0.324	61.11	1.465	0.041	35.73
75	0.040	26.830	0.427	62.83	1.920	0.053	36.23
90	0.039	36.368	0.564	64.48	2.518	0.071	35.46
95	0.039	44.735	0.682	65.59	3.028	0.085	35.62

[a] Data are representative of two separate experiments. Each sample was tested in triplicate, and the mean values are presented. Ratio of the peptides T20 and C34 in combinations is 8:1 (in molecular concentration).

However, unlike C34, which contains the PBD, our previous studies have shown that T20, which lacks the PBD, cannot bind to the hydrophobic pocket region in the C-terminal region of the gp41 CHR domain, the critical site for 6-HB stability, and, thus, is not effective in blocking viral gp41 fusion core formation [21,22]. Since T20 contains the membrane- and N-NHR-binding domains, it may interact with the lipid membrane of the target cell and the N-terminal region of gp41 NHR domain, which contains a GIV motif, a determinant for T20 resistance. This explains why the viruses with mutations in GIV motif are highly resistant to T20, but only moderately resistant to C34 [37]. Based on these findings, we have proposed that T20 inhibits HIV-1 fusion by binding to the N-terminal region of the gp41 NHR domain and the lipid membrane of the target cell, while C34 arrests viral entry by interacting with the C-terminal region of the gp41 NHR domain and blocking viral gp41 6-HB formation. This hypothesis is supported by an abundance of evidence from biophysical and immunological studies [20–22,32,33], but it is not supported by mutation analysis since PBD-containing CHR peptides, such as T2635 and sifuvirtide, could not induce drug-resistance mutations in the gp41 pocket region, but rather at sites far away from the pocket of gp41, or even in some regions of gp120 [50–52]. It is possible that the residues in the pocket region, especially those located at the hydrophobic interface, are too conserved to mutate. Such peptides may induce the mutations of residues outside the gp41 pocket to alter the kinetics of 6-HB formation, thus limiting the time window for these PBD-containing peptides can interfere with membrane fusion [53].

In our previous study, we substituted the residue Gln, a polar residue, at position 64 in the pocket region at the *c* site in the NHR helical wheel with Ala, a smaller amino acid with no polarity (Q64A), or Leu, a hydrophobic residue (Q64L). We found that these mutants retained substantial infectivity, but were highly resistant to CP32M, a PBD-containing CHR peptide [37]. In this study, we used a similar approach to study the resistance mechanism of C34 by selecting residue A67 at the *f* site in the NHR helical wheel for mutations (Figs. 1B and C). We found that viruses with pseudoviruses having A67G mutation in gp41 exhibited high infectivity, while those with A67D and A67S mutations still maintained substantial infectivity (Fig. 2). These three mutants were all highly resistant to C34, but they were relatively sensitive to T20 (Fig. 3 and Table 1). In addition, these mutations had negative effect on α-helical conformation, stability and other biophysical properties of the gp41 6-HB (Figs. 4 and 5). The NHR-peptides with mutations of A67 exhibited decreased α-helicity (Fig. 4A) and reduced anti-HIV-1 activity (Fig. 6). All these changes might contribute to the resistance of A67 mutants to C34.

Since A67 is not located at the hydrophobic interface, change of this residue may not significantly affect the direct interaction between the NHR and CHR. However, this residue may play a role in maintaining the appropriate conformation of the NHR-trimer core formation. Changes of size (A→G) or polarity (A→S) of A67 could affect the conformation of the hydrophobic pocket in the groove of the NHR-trimer, resulting in improper binding of PBD-containing peptides, like C34, and resistance of the viral mutants to C34. These results clearly demonstrated that C34 and T20 target different sites in the gp41 NHR, thus reflecting different mechanisms of action in inhibiting HIV-1 fusion and entry.

Combinatorial use of antiretroviral drugs with different targets or mechanisms of action, such as the highly active antiretroviral therapy (HAART), could significantly increase antiviral potency based on synergism and delay of emerging drug resistance [54]. We previously showed that the combinatorial use of T20 with a PBD-containing CHR peptide, such as T1144 or sifuvirtide, resulted in strong synergistic anti-HIV-1 effect [42,55]. Here, we showed, for the first time, that the combination of T20 with C34 also displayed highly potent synergistic antiviral effect against HIV-1 Env-mediated cell-cell fusion and HIV-1 IIIB infection (Table 3), confirming that T20 and C34 have different target sites of action. Interestingly, the combination of T20 and C34 also exhibited strong synergistic antiviral effect against both T20- and C34-resistant HIV-1 strains (Fig. 7), suggesting a potential regimen for treatment of HIV/AIDS patients who fail to respond to T20 and other HIV entry inhibitors.

In conclusion, using a single-point mutation strategy, we have demonstrated that T20, a CHR-peptide containing MBD and N-NHR-binding domain but lacking PBD, and C34, a PBD-containing CHR-peptide, inhibit HIV-1 fusion by interacting with different target sites. Combining T20 and C34 exhibits strong synergistic antiviral activity against HIV-1 strains, including those resistant to T20 and C34. Therefore, this study provides a theoretical basis for the rational design of novel HIV-1 entry inhibitors and/or more effective regimens for treating patients infected with HIV-1 strains resistant to different HIV entry inhibitors.

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