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Potent anti-HIV-1 activity of N-HR-derived peptides including a deep pocket-forming region without antagonistic effect on T-20

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Abstract

Background—Enfuvirtide (T20), a C-terminal heptad repeat (C-HR)-derived peptide of the human immunodeficiency virus type 1 (HIV-1) glycoprotein, gp41, effectively suppresses HIV-1 replication through a putative mechanism that involves its acting as a decoy and binding to the N-terminal heptad repeat (N-HR) of the virus. In this study, we address whether the anti-HIV-1 activity of T20 is antagonized by a variety of N-HR-derived peptides.

Methods—Multinuclear activation of galactosidase indicator assays were used to evaluate T-20 activity in the presence of N-HR derived peptides. The gp41-derived peptides were chemically synthesized.

Results—We demonstrate additive anti-HIV activity when T20 is used in combination with N-HR-derived peptides that do not have a putative binding region for the tryptophan-rich domain in T20. The presence of a deep pocket-forming region in the N-HR-derived peptides enhanced their anti-HIV-1 activity, but had little impact on the activity of T20.

Conclusion—These results indicate that T20-based antiviral therapies can be combined with N-HR-derived peptides.

Keywords

HIV-1; gp41; fusion inhibitor; combination

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Disclosure statement

The authors declare no competing interests.

Introduction

Two envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1), gp120 and gp41, play an important role in HIV-1 entry into the host cells. The surface subunit gp120 recognizes receptors CD4 and CXCR4/CCR5, which are expressed on the target cell surface. After binding to these receptors, the transmembrane subunit gp41 (Fig. 1A) mediates fusion of HIV-1 to the host cells. Viral membrane-anchored gp41 forms a 6-helix bundle composed of an α -helical trimer of an N-terminal heptad repeat (N-HR) with three C-terminal heptad repeats (C-HR) folded onto the N-HR in an anti-parallel orientation [1]. Formation of the 6-helix bundle enables fusion between the viral and the host cell membranes. Therefore, peptides derived from either HIV-1 gp41 N- or C-HR sequences have been thought to inhibit HIV-1 fusion by preventing formation of the 6-helix bundle, and by acting as a dominant negative or a decoy [2, 3].

C34 is a representative C-HR-derived peptide that inhibits HIV-1 fusion to host cells. It contains four key amino acids, W117, W120, D121, and I124, collectively designated as the N-terminal tryptophan-rich domain (N-TRD), which dock into a hydrophobic pocket (termed the “deep pocket”) formed by the N-HR trimer (Fig. 1B) [4]. Enfuvirtide (T20), which lacks 10 amino acids in the N-terminus that are present in C34, also contains four key amino acids, W159, N160, W161, and F162, which are collectively designated as the C-terminal tryptophan-rich domain (C-TRD). Both TRDs have been demonstrated to be important for inhibition of HIV-1 fusion [5–10]. In a previous study, we used enzyme linked immunosorbent assay (ELISA) to demonstrate that T20 binds weakly to the N-HR, even in the case of N-HR containing the entire T20 binding region with amino acid position from 18 to 73, whereas C34 binds strongly [11]. Our previous findings are consistent with other observations that T20 may only transiently interact with N-HR and instead, it inhibits HIV-1 fusion by targeting multiple sites in both gp120 and gp41 (Liu et al., 2005). However, T20 has been shown to interact with an artificial 5-helix complex comprised of a linker-connected structure containing three N-HRs and two C-HRs, although the interaction was much weaker than that between C34 and the complex [12]. Hence, the C-TRD of T20 may have a distinct role during the inhibition of HIV-1 fusion, other than binding to the N-HR. A mechanism that involves membrane association of T20 *via* C-TRD has been previously proposed for the fusion inhibition [9, 13, 14].

In contrast to the results from extensive biochemical analyses, the results accumulated from virological experiments support the hypothesis that T20 interacts with the N-HR region. Hence, the majority of T20-resistant HIV-1 variants acquire mutations in the N-HR, especially from L33 to L45, which is the putative interactive site of T20 (Fig. 1B) [15–23]. Moreover, the inhibitory effect of T20 seems to depend on the specific sequence of the N-HR (Liu et al., 2005; Nishikawa et al., 2009). Therefore, the avidity of T20 with N-HR remains unclear. We hypothesized that any potential interactions of T20 with N-HR-derived peptides would attenuate its anti-HIV-1 activity by adsorption into the added N-HR-derived peptides, which form an inactive 6-helix bundle. In this study, using synthesized various N-HR-derived peptides with or without the putative T20 binding region, we determined the anti-HIV-1 activity of T20 and C34 in the presence of the synthesized peptides. Our data

identify additive combinations of T20 and N-HR-derived peptides, which may be valuable for treating HIV-1.

Methods

Cells and viruses

293T cells were grown in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, and 50 µg/mL streptomycin. HeLa-CD4/CCR5-LTR/β-gal cells [24] (provided by Dr. J. Overbaugh through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID) were maintained in DMEM supplemented with 10% FCS, 200 µg/mL hygromycin B, 10 µg/mL puromycin, and 200 µg/mL geneticin. Wild-type HIV-1 was harvested from the supernatant of an HIV-1 molecular clone, pNL4-3 transfected 293T cells and stored at −80°C.

Determination of drug susceptibility

Anti-HIV activity of inhibitors was determined using multinuclear activation of a galactosidase indicator (MAGI) assay as described previously [19, 25–30]. Briefly, HeLa-CD4-LTR-β-gal cells were inoculated with HIV-1_{NL4-3} (60 MAGI units, generating 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of peptides. Forty-eight hours after initial viral exposure, we counted all the blue cells that were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We assessed the antiviral activity of peptides based on the effective concentration that blocked HIV-1 infection by 50% (EC₅₀).

Statistical analysis

Dunnett's multiple comparisons test was used to evaluate whether the EC₅₀ values of T-20 were influenced in the presence of N-HR peptides. *P*-values less than 0.01 were considered statistically significant.

Results

Antiviral activity of the N-HR-derived peptides

Six N-HR-derived peptides, N36, N36-T, N46, N43-T, N45-T, and N54, were synthesized with standard Fmoc-based solid-phase techniques (Fig. 1B) [31]. We first examined whether these N-HR-derived peptides exerted anti-HIV-1 activity with the MAGI assay [27]. We found that N36, N46, and N54, which include the entire deep pocket-forming region corresponding to N-TRD, were more active than N36-T, N43-T, and N45-T, which include only part of the region (Fig. 1B). However, addition of the region of N-HR that is believed to interact with C-TRD to the N-HR derived peptides had little influence on the activity of the N-HR-derived peptides. These results suggest that the addition of a deep pocket-forming region to N-HR-derived peptides provides enhanced antiviral activity of the peptides.

Combination of T20 and N-HR-derived peptides with a deep pocket-forming region

We also tested whether the anti-HIV-1 activity of T20 is attenuated when used in combination with the N-HR-derived peptides (Fig. 2). Specifically, we examined the antiviral activity of T20 or C34 at concentrations ranging from 0.1 to 100 nM, in the presence or absence of each N-HR-derived peptide (concentration at 30–40% inhibition of HIV replication), which provided apparent effect of the combination among other concentrations tested. We found additive effect in combination of T20 and N36, N36-T, or N46, which lack the interactive site for C-TRD in T20 (Fig. 2A). In contrast, the activity of C34 was decreased to the level of C34 alone in the presence of N36, N36T or N46, respectively, which contains the N-TRD corresponding region (Fig. C). Moreover, C34-mediated inhibition of the fusion was attenuated especially by N46 and N54 at 10 nM C34 ($p < 0.01$, Fig. 2C, D), indicating that a deep pocket region is important for a reduction in the C34 activity, possibly by a direct interaction (adsorption) with N-HR. Although N36-T and N46 include part of the C-TRD binding region, the avidity of the T20 complex with these peptides seemed to be vulnerable in the fusion between HIV-1 and the host cells. Therefore, we further examined the N-HR-derived peptides that contain an entire interactive region of the C-TRD.

Role of C-TRD corresponding region in the N-HR-derived peptides

The combination studies of T20 revealed that the antiviral activity of N43-T or N45-T, but not of N54, which includes both the N- and C-TRD corresponding regions, was reduced to levels comparable to T20 alone, indicating antagonism (Fig. 1 and 2B). However, the antiviral activity of C34 was significantly antagonized by either N46 or N54 at 10 nM (Fig. 2C, D). These results indicate that putative interactive region in the N-HR-derived peptides for C-TRD plays small role in T20 binding.

Discussion

In this study, we address possible combination of T20 with the N-HR-derived peptides through their influence on T20 activity. To date, the mechanisms of T20 action have been extensively examined in protein-protein in vitro binding but rarely in actual HIV-1 entry. Previously, Liu *et al.* also tried to reveal a role of the C-TRD in T20 activity by T20-ANAA, which is like traditional T20 but with a substitution from WNWF to ANAA in the C-TRD [14]. T20-ANAA exhibited 450-fold less anti-HIV-1 activity than T20. However, it was shown that the moderate levels of T20-ANAA avidities toward N36-F10 [14] and the 5-helix bundle [12] were maintained, suggesting that the WNWF motif is unnecessary for binding to N-HR, but is important for potent anti-HIV-1 activity of T20. Indeed, the overall reduction in T20 activity by the N-HR peptides with the C-TRD corresponding region was moderate. The anti-HIV-1 activity of T20 was not blocked by combination with N54, which includes both the C-TRD binding region and the deep pocket-forming region. The latter region may enhance the activity of the N-HR peptides and restore the antagonistic effect on T20.

We clearly demonstrated that unlike the C-TRD, which only weakly interacts with the N-HR, N-TRD, is definitively required for the interaction with the N-HR, thus playing a

distinct role in HIV-1 fusion. Recently, it was reported that an N-HR-derived peptide lacking a binding site for the TRD of T20, (CCIZN17)₃, which is covalently stabilized by three interchain disulfides, can be effectively combined with T20 [32]. To design sustained anti-HIV-1 activity of N-HR-derived peptides in combination with T20, the amino acid sequence at positions 60–70 that forms a deep pocket should be included in the peptide and the N-terminal sequence of N-HR should be excluded. Our observations should be useful for further development of potent and N-HR-derived peptides that could be effectively combined with T20.

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Abbreviations

HIV-1	human immunodeficiency virus type 1
N-HR	N-terminal heptad repeat
C-HR	C-terminal heptad repeat
ELISA	enzyme-linked immunosorbent assay
MAGI	multinuclear activation of a β -galactosidase indicator
TRD	tryptophan-rich domain
T20	enfuvirtide
N-TRD	N-terminal tryptophan-rich domain
C-TRD	C-terminal tryptophan-rich domain
EC₅₀	50% effective concentration

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Figure 1.

Schematic view of HIV-1 gp41 (A) and the amino acid sequence of gp41-derived peptides, in addition to the inhibitory effect of the N-terminal heptad repeat (N-HR)-derived peptides (B). (A) The locations of the fusion peptide (FP), the N-HR region (yellow), the C-terminal heptad repeat region (C-HR; light blue), and the transmembrane domain (TM). (B) The relationship between the N- and C-HR regions when gp41 is folded over into a hairpin [33] and the peptides used in this study. The residue numbers of each peptide correspond to their positions in HIV-1 gp41. The EC₅₀ of N- and C-HR-derived peptides for inhibition of HIV-1_{NL4-3} were obtained using the MAGI assay. The hydrophobic deep pocket-forming sequence in the N-HR is indicated in blue. Four key amino acids, W117, W120, D121, and I124, which are designated as the N-terminal tryptophan-rich domain (N-TRD) of C-HR, dock into the deep pocket of the trimer of N-HR. The amino acids W159, N160, W161, and F162, which are designated as the C-terminal tryptophan-rich domain (C-TRD), are indicated in red.

Figure 2.

Inhibition curves of the combinations of T20 (A, B) or C34 with the individual N-HR-derived peptides (C, D) determined with the MAGI assay. The data represent the average percentage of a no-reagent control blue cell-forming unit (BFU) and the standard deviation obtained from the results of at least five independent experiments. The anti-HIV-1 activities of enfuvirtide (T20) (A) and C34 (C) in the absence and presence of the N-HR-derived peptides N36 (0.3 μ M), N36-T (0.8 μ M), and N46 (0.125 μ M), which include a deep pocket-forming region. In contrast, N43-T (1.0 μ M), N45-T (1.5 μ M), and N54 (0.15 μ M) include a putative interactive region for C-TRD. Asterisks indicate a significant decrease in the entry inhibition according to Dunnett's multiple comparisons test ($p < 0.01$).