A Novel Chimeric Protein-based HIV-1 Fusion Inhibitor Targeting gp41 Glycoprotein with High Potency and Stability*

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T20 (enfuvirtide, Fuzeon) is the first generation HIV-1 fusion inhibitor approved for salvage therapy of HIV-1-infected patients refractory to current antiretroviral drugs. However, its application is limited by the high cost of peptide synthesis, rapid proteolysis, and poor efficacy against emerging drug-resistant strains. Here we reported the design of a novel chimera protein-based fusion inhibitor targeting gp41, TLT35, that uses a flexible 35-mer linker to couple T20 and T1144, the first and next generation HIV-1 fusion inhibitors, respectively. TLT35, which was expressed in Escherichia coli with good yield, showed low nM activity against HIV-1-mediated cell-cell fusion and infection by laboratory-adapted HIV-1 strains (X4 or R5), including T20-resistant variants and primary HIV-1 isolates of clades A to G and group O (R5 or X4R5). TLT35 was stable in human sera and in peripheral blood mononuclear cell culture and was more resistant to proteolysis than either T20 or T1144 alone. Circular dichroism spectra showed that TLT35 folded into a thermally stable conformation with high α-helical content and Tm value in aqueous solution. It formed a highly stable complex with gp41 N-terminal heptad repeat peptide and blocked formation of the gp41 six-helix-bundle core. These merits combined with an anticipated low production cost for expression of TLT35 in E. coli make this novel protein-based fusion inhibitor a promising candidate for further development as an anti-HIV-1 microbicide or therapeutic for the prevention and treatment of HIV-1 infection.

Human immunodeficiency virus type 1(HIV-1) enters and infects host cells through envelope protein-mediated viral and host cell membrane fusion (1, 2). HIV-1 envelope protein is a complex of non-covalent-associated gp120 and gp41, the surface and transmembrane subunits, respectively. The fusion process is initiated by the binding of gp120 to the cellular surface receptor CD4 and a co-receptor, CCR5 or CXCR4, triggering a cascade of large conformational changes of gp120/gp41 complex from a native state to a prehairpin fusion intermediate state and then to a hairpin fusion state (3–5). The fusion core formed at the fusion state contains a six-helical bundle (6-HB) in which three gp41 N-terminal heptad repeats (NHR) form a trimeric inner core and three C-terminal heptad repeats (CHR) pack in an antiparallel manner against the inner trimer (6–8). The free energy released by bundle formation drives the apposition and subsequent fusion of viral and target cell membranes (9). Peptides derived from the NHR and CHR sequences can bind to their counterparts in gp41 to form heterotrimers and prevent the viral gp41 6-HB core formation, resulting in inhibition of HIV-1 fusion and entry into the target cell (10–12).

T20 (generic name, enfuvirtide; brand name, Fuzeon) is the first generation HIV-1 fusion inhibitor approved for salvage therapy of HIV-1-infected patients refractory to current antiretroviral drugs (13, 14). Its application has been limited by (i) the high cost of synthesis peptide, (ii) rapid proteolysis, and (iii) poor efficacy against emerging T20-resistant strains. These drawbacks called for a new generation of fusion inhibitors with improved antiviral and pharmacokinetic profiles. In response, researchers at Trimeris designed and developed the next generation HIV-1 fusion inhibitors, T1249 (15) and T1144 (16), respectively.

Here we report the development of a chimera protein-based HIV-1 fusion inhibitor, designated TLT35, that employs a 35-mer linker to couple T1144, a 38-mer CHR-peptide, which contains a heptad-repeat binding domain (HBD) and a pocket binding domain (PBD), and T20, which contains a HBD and a lipid binding domain (LBD) (Fig. 1). The optimized chimera polypeptide has a sequence with ~110 amino acid residues, making it suitable for recombinant expression (17). We found that TLT35 was expressed as a soluble protein with highly potent anti-HIV-1 activity against laboratory-adapted and primary HIV-1 strains, including T20-resistant mutant. TLT35 folds into a thermally stable conformation with high α-helical content and Tm value in aqueous solution. It formed a highly stable complex with gp41 NHR peptide and blocked formation of the gp41 6-HB core. TLT35 was very stable in human sera and peripheral blood mononuclear cell (PBMC) culture and was more resistant to proteolysis than its parent peptides.

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3 The abbreviations used are: 6-HB, six-helix bundle; NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; HBD, heptad-repeat binding domain, PBD, pocket binding domain; LBD, lipid binding domain; PBMC, peripheral blood mononuclear cell; N-PAGE, native PAGE; FN-PAGE, fluorescence native PAGE; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; FAM, 6-carboxyfluorescein.
TLT35 was sufficiently expressed in *Escherichia coli* for large-scale manufacture. All these merits suggest that TLT35 is a promising candidate for the development of a new generation HIV-1 fusion inhibitor.

**EXPERIMENTAL PROCEDURES**

**Reagents**—MT-2, TZM-bl, and HIV-1 IIIB-infected H9 (H9/HIV-1 IIIB) cells as well as HIV-1 strains were obtained from the NIH AIDS Research and Reference Reagent Program. Peptides T20, T1144, C34-FAM, N36, and N46 (Fig. 1) were synthesized by a standard solid-phase Fmoc (N-(9-fluorenyl) methoxycarbonyl) method using an Applied Biosystems Model 433A peptide synthesizer. All peptides were acetylated at the N termini and amidated at the C termini. The peptides were purified to homogeneity (>95% purity) by high performance liquid chromatography and identified by laser desorption mass spectrometry (PerSeptive Biosystems, Framingham, MA). The peptide concentration was determined according to Edelhoch (18) by measuring UV absorbance of the peptide in 6 M guanidine hydrochloride solution and calculating the molar extinction coefficient (280 nm) of 5500 and 1490 mol/liter cm based on the number of Trp residues and Tyr residues (all the peptides tested contain Trp and/or Tyr), respectively.

**Construction of the Expression Vectors**—To create the expression plasmid pTLTx (x means the number of amino acids in the linker, as shown in Table 1), the DNA fragments encoding T1144, the linker (Lx), and T20 were linked together by three-step overlapping PCR. First, the T1144, Lx, and T20 DNA fragments were generated by overlapping PCR using the corresponding primer pairs as described in Table 2. Second, the DNA fragments coding for Lx and T20 were linked by overlapping PCR with the primers FLx and RT20. Third, the two DNA fragments encoding T1144 and Lx-T20 were linked by overlapping PCR with the primers FT1144 and RT20. Finally, the amplified DNA fragment coding for T1144-Lx-T20 was digested by BamHI and XhoI and inserted into the expression vector pGEX-6p-1 to generate the pTLTx plasmid. The plasmid was produced in *E. coli* DH5α and the inserted sequences were checked by sequencing.

**Production of Peptide Chimeras**—To express the GST-TLTx fusion proteins, *E. coli* strain Rosetta was transfected with the plasmid pTLxT. The stable clones with the highest protein expression level were selected and expanded for overproduction of TLTx. The bacteria were lysed with PBS (pH 7.2) using sonication. After centrifugation, the supernatants containing the fusion protein were collected. The GST-TLTx fusion proteins were then purified by glutathione-Sepharose 4B affinity columns and treated with PreScission™ Protease to release the chimeras from GST. Purified homogeneity was obtained by fast protein liquid chromatography (FPLC) and analyzed by SDS-PAGE with Novex® 10–20% Tricine gel. The yield of TLTx was determined on Thermo NanoDrop ND-1000.

**Circular Dichroism (CD)**—CD measurements were performed as previously described (19, 20). Briefly, the peptides were dissolved in PBS (50 mm sodium phosphate and 150 mm NaCl (pH 7.2)). Individual peptides at 8 μM or mixtures of 8 μM concentrations of each peptide in PBS were incubated at 37 °C for 30 min. The CD spectrum of each sample was acquired on a...
TABLE 2

| Primer | Gene | Sequence(5’/H1154 to 3’/H1154) |
|--------|------|----------------------------------|
| T1144  | FT1144 | CCGGGGATCCAcgacctggGAAGCAtgggacagaGCTattGCTGAAtacGCAGCTAGGataGAAGCTttACTCAGAGCTTTA |
| RT1144 | ACTTCCTCCTCCTCCTAATTCCCTTAAGGCTGCTTCATTCTTTTCTTGCTGTTCTTGTAAAGCTCTGAGTAAAGC |
| GGAGGCGGAGGTAGCtacacaagcttaatacactccttAattgaagaatcgcaaaaccagcaagaaaagaatgaacaa |
| FL10  | T20  | CCGCTCGAGTTAAAACCAATTCCACAAACTTGCCCATTTATCTAATTCCAATAATTCTTGTTCATTCTTTTC |
| RT20  | GGAGGAGGAGGAAGTGGAGGCGGAGGTAGC |
| 10-Mer linker | FL10 | GGAGGAGGAGGAAGTGGCGGCGGCGGCTCGGGAGGCGGAGGTAGC |
| 15-Mer linker | FL15 | GGAGGAGGAGGAAGTGGCGGCGGCGGCTCGGGTGGTGGTGGTTCTGGAGGCGGAGGTAGC |
| 20-Mer linker | FL20 | GGAGGAGGAGGAAGTGGCGGCGGCGGCTCGGGTGGTGGTGGTTCTGGAGGT |
| 25-Mer linker | FL25 | GGAGGAGGAGGAAGTGGCGGCGGCGGCTCGGGTGGTGGTGGTTCTGGAGGT |
| 30-Mer linker | FL35 | GGAGGAGGAGGAAGTGGCGGCGGCGGCTCGGGTGGTGGTGGTTCTGGAGGTGGCGGTAGCGGAGGTGGAGGTAGTGGAGGC |
| 35-Mer linker | RL35 | GCTACCTCCGCCTCCGACACCTCCGCCTCCACTACCTCCACCTCCGCTACCGCCACCTCCAGAACCACC |
| 40-Mer Linker | FL35 | GGAGGAGGAGGAAGTGGCGGCGGCGGCTCGGGTGGTGGTGGTTCTGGAGGTGGCGGTAGCGGAGGTGGAGGTAGTGGAGGC |
| RL40  | GCTACCTCCGCCTCCAGAACCGGAGCCTGACGAACCTCCGCCTCCACTACCTCC |

The underlined portions of the sequence are restriction enzyme sites used for cloning a gene into vector pGEX-6p-1.

A Chimeric Protein-based HIV-1 Fusion Inhibitor Targeting gp41

Jasco spectropolarimeter (Model J-715, Jasco Inc.) at 20 °C using a 5-nm bandwidth, 0.5-nm resolution, 0.1-cm path length, and an average time of 5.0 s. Spectra were corrected by the subtraction of a blank corresponding to the solvent composition of each sample. The α-helical content was calculated from the CD signal using the K2D program (21). Thermal denaturation was monitored at 222 nm by applying a thermal gradient of 2 °C/min in the range of 20–98 °C. To determine the reversibility, the peptide mixtures were cooled to room temperature after a thermal scan and kept for 30 min, after which spectra were re-measured and compared with those before thermal denaturation. The melting curve was smoothed, and the midpoint of the thermal unfolding transition (Tm) values was calculated using Jasco software utilities.

Native Polyacrylamide Gel Electrophoresis (N-PAGE)—N-PAGE was carried out to determine the 6-HB formation between the N- and C-peptides, as described previously (20). Briefly, TLTx, C-peptide alone or mixtures with same molar of N-peptide (N36) at a final concentration of 40 μM were incubated at 37 °C for 30 min. The peptide samples were mixed with the same volume of 2× Tris-glycine native sample buffer (Invitrogen), and the mixture was loaded onto 10 × 1.0-cm precast 18% Tris-glycine gels (Invitrogen) at 25 μl/ per well. Gel electrophoresis was carried out with 125 V of constant voltage at room temperature for 2 h. The gel was then stained with Coomassie Blue and imaged with a FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA).

Fluorescence Native Polyacrylamide Gel Electrophoresis (FN-PAGE)—FN-PAGE was performed under the same conditions using the same reagents as those for N-PAGE described above, except that FITC-conjugated peptide C34-F was added to block 6-HB formation by N and C peptides (19). Immediately after electrophoresis, fluorescence bands in the gel were imaged by the FluorChem 8800 Imaging System using a transillumination UV light source with an excitation wavelength at 302 nm and a fluorescence filter with an emission wavelength at 520 nm. The gel was then stained with Coomassie Blue and imaged again with a FluorChem 8800 Imaging System.

Western Blotting—Peptides were run at 10 μg/sample in SDS Novex® 10–20% Tricine gel and then transferred onto PVDF membranes. Primary antibodies were mixed by mouse anti-T1144 and mouse anti-T20 at 10 μg/ml for each antibody. Secondary antibody was alkaline phosphatase-conjugated goat anti-mouse IgG. The blot was developed by using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

HIV-1-mediated Cell-Cell Fusion—A dye transfer assay was used for detection of HIV-1-mediated cell-cell fusion as previously described (22, 23). Briefly, calcein-AM-labeled H9/HIV-1IIIb-infected cells were incubated with MT-2 cells (ratio = 1:5) at 37 °C for 2 h in the presence or absence of the test peptide. The fused and unfused calcein-labeled HIV-1-infected cells were counted under an inverted fluorescence microscope (Zeiss) with an eyepiece micrometer disc. The percent inhibition of cell-cell fusion and the IC50 values were calculated as described before (24).

Measurement of Anti-HIV-1 Activity against Laboratory-adapted HIV-1 Strains and T20-resistant Viruses—The inhibitory activity of the peptide HIV-1 fusion inhibitors on infection
by laboratory-adapted HIV-1 strains and T20-resistant viruses was determined as previously described (24, 25). In brief, 1 × 10⁴ MT-2 cells were infected with HIV-1 isolates at 100 TCID₅₀ (50% tissue culture infective dose) overnight in 200 μl of RPMI 1640 medium containing 10% fetal bovine serum in the presence or absence of the test peptide at graded concentration. Then the culture supernatants were removed, and fresh media were added without inhibitors. 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 assayed for p24 antigen by ELISA as previously described (24).

**Determination of Inhibition of Infection by Primary HIV-1 Isolates**—The inhibitory activity of the peptide HIV-1 fusion inhibitors against a primary HIV-1 isolate was determined as previously described (24, 26). Briefly, PBMCs were isolated from the blood of healthy donors using a standard density gradient (Histopaque-1077, Sigma) centrifugation. The cells were plated in 75-cm² plastic flasks and incubated at 37 °C for 2 h. The nonadherent cells were collected and resuspended at 5 × 10⁵/ml in RPMI 1640 medium containing 10% FBS, 5 μg of phytohemagglutinin/ml, and 100 units of interleukin-2/ml followed by incubation at 37 °C for 3 days. The phytohemagglutinin-stimulated cells were infected with a primary HIV-1 isolate at a multiplicity of infection of 0.01 in the absence or presence of a peptide at graded concentrations. The supernatants were collected 7 days post-infection and tested for p24 antigen by ELISA. The percent inhibition of p24 production and IC₅₀ values were calculated.

**Stability Study**—To test the sensitivities of T20, T1144, and TLT35 to the proteolytic effect of proteinase K and pepsin, 4 μM concentrations of T20, T1144, and TLT35 were dissolved in PBS and then incubated with either proteinase K or pepsin beads containing 0.1 unit/ml proteinase K or 10 unit/ml pepsin, respectively, at 37 °C. At 0, 5, 10, 20, 40, and 60 min, samples were collected and kept at −20 °C. To test the stability of these peptides stored at different temperatures for different time periods, T20, T1144, and TLT35 were dissolved in PBS (4 μM) and maintained at 4, 25, and 37 °C for 1 week. On each day the
samples were collected and kept at −20 °C before testing their residual inhibitory activity against HIV-1-mediated cell-cell fusion activity.

Immunogenicity Test—Balb/c mice were subcutaneously immunized with 10 µg of TLT35, T20, and T1144 in the absence of adjuvant and boosted twice at 10-day intervals with the same amount of the corresponding antigen. Mice were bled 10 days after last boost, and antibodies in the sera against the respective inhibitors were tested by ELISA.

RESULTS

Design, Expression, and Purification of the Chimera TLTx Proteins—To design the chimera polypeptide, T20 and T1144 were selected. T20, the first generation fusion inhibitor derived from the gp41 CHR region, contains HBD, including a GIV motif that is the determinant for T20-resistance, and LBD. T1144, the third generation fusion inhibitor, contains HBD and PBD that can interact with a highly conserved hydrophobic deep pocket formed by the pocket-forming sequence in the C-terminal region of the gp41 NHR domain (Fig. 1A), which plays a critical role in maintaining the stability of the 6-HB core of gp41 and viral fusion (27–29). We expected that the selected peptide fusion inhibitors with different functional domains would have a complementary effect and that the difference in sequence origin between them would enhance the chance of their interaction in the chimera protein. The two peptides were linked by a flexible (GGGGS)ₙ amino acid linker, and various linker lengths were designed for structure-active optimization (Fig. 1B). These chimera peptides were designated TLTx, where x indicates the length (the number of the amino acid residues) of the linker. TLTx was designed to fuse with GST protein to facilitate expression and purification.

The vectors encoding the designed TLTx sequences were constructed using standard molecular biology protocol. The vectors bearing correct TLTx sequences were transferred into E. coli for overexpression as soluble protein, which was then purified by glutathione-Sepharose 4B affinity column. After enzymatic cleavage, TLT was released and purified to homogeneity by FPLC. The final TLTx contained an additional 5 residues, GPLGS, from enzymatic cleavage. The purity and molecular size (8–11 kDa) of TLTx was confirmed by SDS-PAGE (Fig. 1C).

TLTx Forms Stable 6-HB with N-peptide and Prevents the gp41 Fusion Core Formation—N-PAGE combined with CD spectra provides a useful tool to characterize 6-HB formation. The band of NHR-peptide (N36, line 1 in Fig. 2A) did not show

FIGURE 2. Interaction of TLTx with N36 as determined by N-PAGE. A, N-PAGE analysis of 6-HB formation by N36 and T1144, T20, and TLTx is shown. B, shown is inhibition of 6-HB formation between N36 and C34-FAM by T1144, T20, and TLTx, as determined by FN-PAGE (a) and N-PAGE (b).

TABLE 3

HIV-1 fusion inhibitory activity and protein yield of TLTx

The experiments were performed in triplicate, and the data are presented as the mean ± S.D.

| Peptides | IC₅₀ for inhibiting cell fusion | Yield of peptide |
|----------|-------------------------------|-----------------|
|          | µM                           | µg/liter        |
| TLT10    | 28.70 ± 2.42                 | 511 ± 361       |
| TLT15    | 19.89 ± 1.63                 | 693 ± 155       |
| TLT20    | 11.71 ± 0.98                 | 809 ± 209       |
| TLT25    | 10.74 ± 0.73                 | 795 ± 173       |
| TLT30    | 6.36 ± 1.33                  | 856 ± 124       |
| TLT35    | 3.98 ± 0.73                  | 1,841 ± 730     |
| TLT40    | 25.54 ± 3.78                 | 1,927 ± 1,015   |

* Indicates the length (the number of the amino acid residues) of the linker. TLTx was designed to fuse with GST protein to facilitate expression and purification.


TABLE 4

Inhibitory activity of TLT35 on infection by laboratory-adapted and primary HIV-1 strains

The experiments were performed in triplicate, and the data are presented as the mean ± S.D.

| HIV-1 strains | T20 | T1144 | TLT35 |
|--------------|-----|-------|-------|
| IIIB (B, X4) | 66.75 ± 11.90 | 16.17 ± 2.25 | 11.06 ± 3.12 |
| Bal (B, R5)  | 13.86 ± 4.76  | 5.46 ± 0.5   | 2.24 ± 0.68  |
| NL4-3 (V38A, N42G) | >500 | 4.17 ± 0.63 | 3.98 ± 0.88 |
| 92UG103 (A, X4R5) | 33.76 ± 2.78 | 14.31 ± 2.85 | 8.89 ± 1.29 |
| 92US677 (B, R5)  | 45.89 ± 4.29  | 14.16 ± 1.13 | 5.76 ± 0.67  |
| 93MWH595 (C, R5) | 3.70 ± 0.57   | 5.70 ± 0.37  | 3.61 ± 0.11  |
| CMU02 (E, R5)   | 23.98 ± 0.55  | 36.44 ± 1.08 | 3.65 ± 0.57  |
| 93TH051 (E, X4R5) | 7.01 ± 1.79   | 5.82 ± 1.06  | 2.55 ± 0.78  |
| 93BR020 (F, X4R5) | 33.34 ± 12.98 | 4.00 ± 0.67  | 1.83 ± 0.58  |
| RU570 (G, R5)   | 7.63 ± 0.001  | 19.29 ± 1.16 | 5.15 ± 0.39  |
| BCF02 (group O, R5) | 5.52 ± 0.20  | 27.87 ± 1.02 | 14.89 ± 1.08 |

* A T20-resistant strain.

TLT35 Exhibited the Best HIV-1 Fusion Inhibitory Activity and Protein Yield—The inhibitory activity of the purified TLTx against HIV-1-mediated cell-cell fusion was determined by a dye transfer assay (22, 23). As shown in Table 3, the HIV-1 fusion inhibitory activity of TLTx increased with the increasing length of the linker between T1144 and T20, reaching maximum activity at TLT35, with the activity decreasing thereafter. Interestingly, the production yield of the recombinant TLTxs was also increased as the length of the linker increased and reached a maximum at TLT35. Therefore, TLT35 was selected for further studies.

TLT35 Folded into Structured Proteins with a High α-Helix Content and Formed Highly Thermally Stable Complexes with N-peptide—Circular dichroism spectra (CD) were used to study the secondary structure of T20, T1144, and TLT35 as well as their complex formed with an NHR peptide, N36. T20 is unstructured in solution (14% α-helix), whereas T1144 forms a typical α-helical structure with calculated 100% helicity in solution using K2D program (21). TLT35 also forms typical α-helical structure with a calculated 70% helical content in solution (Fig. 3A), which corresponds to 103% helical content for T1144 + T20 sequence if we assume that the 35-mer linker adapts a random coil structure in TLT. This indicated that both T1144 and T20 folded into a well structured functional domain in the chimera, in striking contrast to the unstructured character of T20 under the same conditions. To check the interaction induced by the secondary structure change of the two peptides, we measured the CD spectra of the mixtures of these CHR peptides and N36. Consistent with our previous observation, T20/N36 interaction did not induce an α-helical structure (29), whereas T1144/N36 interaction induced a large amount of α-helix and formed stable α-
helical complexes (Fig. 3B). Like T1144, the chimera protein TLT35 bound tightly to N36 and formed stable complexes with $T_m > 80$ °C in PBS (Fig. 3C).

**TLT35 Was Highly Effective against a Broad Spectrum of HIV-1 Strains, Including Laboratory-adapted and Primary HIV-1 Strains as Well as the T20-resistant HIV-1 Strain**—Then we tested the activity of TLT35 along with T20 and T1144 against infection by various HIV-1 strains, including primary and laboratory-adapted isolates spanning classes A, B, C, E, F, G, and group O, with R5, X4, or R5/X4 dual tropism. TLT35 showed low $nM$ antiviral breadth against most of the tested HIV-1 strains, including some R5 strains, which were less sensitive to T1144 (Table 4). It is worth noting that TLT35 was more potent against the resistant strains, including T20-resistant strain NL4-3 (V38A, N42G) and T1144-insensitive strain CMU02 (E, R5), which showed a 10–100-fold increase in potency (Table 4). Therefore, TLT35 is suitable as a next generation HIV-1 fusion inhibitor to treat patients who fail to respond to T20 treatment.

**TABLE 5**

Stability of TLT35 and its sensitivity to proteolytic enzymes

| Peptide cultured with | Peptide | Half-life (min) | -Fold |
|-----------------------|---------|----------------|-------|
| Proteinase K          | T20     | 2.5            | 1     |
| Proteinase K          | T1144   | 18.0           | 7.2   |
| Proteinase K          | TLT35   | 18.3           | 7.3   |
| Pepsin                | T20     | 1.4            | 1     |
| Pepsin                | T1144   | 13.9           | 8.9   |
| Pepsin                | TLT35   | 48.9           | 34.9  |
| PBMCs                 | T20     | 8.4            | 1     |
| PBMCs                 | T1144   | >150           | >17.9 |
| PBMCs                 | TLT35   | >150           | >17.9 |

**FIGURE 4. Stability of TLT35 and its sensitivity to proteolytic enzymes.** Sensitivity of TLT35 to proteinase K treatment (A) is shown. Sensitivity of TLT35 to pepsin treatment (B) is shown. Stability of TLT35 in PBMC cultures (C) is shown. Stability of TLT35 in PBS when stored at 4 °C (D), 25 °C (E), and 37 °C (F) for different time periods, respectively, is shown. The residual anti-HIV-1 activity was determined using the HIV-1-mediated cell-cell fusion assay. Each sample was tested in triplicate and was presented in mean ± S.D.
TLT35 Was More Resistant to Proteolysis and More Stable in PBMC Culture and PBS than T20 When Stored at Different Temperatures for Different Time Periods—We tested the stability of TLT35 by proteinase K and pepsin treatments (Fig. 4, A and B, respectively) and its stability in PBMC culture (Fig. 4 C), and we compared the results with those of T20 and T1144. TLT35 was more resistant to pepsin treatment than T20 and T1144. Its stability was equal to that of T1144 but more stable than T20 in PBMC culture and the proteinase K test (Table 5). Like T1144, TLT35 is much more stable than T20 when stored at 4 °C (Fig. 4D), 25 °C (Fig. 4E), and 37 °C (Fig. 4F). The stability of TLT35 may have resulted from its well folded structure, which shields its proteolytic site.

TLT35 Showed Weak Immunogenicity When Administered in the Absence of Adjuvant and Resistance to Anti-TLT35 Antibodies—TLT35, a well folded protein with an Mr around 12 kDa, may induce antibodies after it is used in vivo in human, which may block the anti-HIV-1 activity of TLT35. Therefore, it is necessary to test its immunogenicity. Mice were subcutaneously administered with T20, T1144, and TLT35 in the absence of adjuvant in a way similar to that for using T20 in clinics and boosted twice after 10 days. The antibody responses against the corresponding peptide or protein were evaluated 30 days after the first immunization. Very low titers of the antibodies were observed for T20 (1:80), T1144 (1:80), and TLT35 (1:40) (Fig. 5A). Then, we assessed the cell fusion inhibitory
activity of TLT35 after it was treated with the antisera at different dilution. As shown in Fig. 5B, the inhibitory activity of these inhibitors against HIV-1-mediated cell-cell fusion was not blocked by the corresponding antisera at the concentration as high as 1:20. These results suggest that because of the low immunogenicity, TLT35 can only induce low level antibody response and the anti-TLT35 antibodies cannot significantly inhibit TLT35-mediated anti-HIV-1 activity.

**DISCUSSION**

To design chimera proteins able to overcome the insolubility and instability of separated NHR and CHR peptide fusion inhibitors, two or more pieces of NHR or CHR peptides were linked to form a stable structure in physiological condition. The chimera fusion inhibitors we engineered usually contain >100 amino acid residues and are suitable for production as recombinant protein, thus avoiding the otherwise high costs associated with peptide synthesis (31, 32). Particularly, the chimera protein fusion inhibitor TLT35 is composed of two CHR peptides, T20 and T1144, the first and next generation of fusion inhibitors. TLT35 was successfully expressed in *E. coli* with good yield. It folded into a stable structure with high α-helical content and high *T_m*; it bound tightly with NHR and formed a highly thermally stable complex. Accordingly, TLT35 strongly inhibited 6-HB formation and was, consequently, highly active against HIV-1 gp41-mediated cell-cell fusion.

TLT35 showed low nanomolar activity against infection by various HIV-1 strains, and it was more active than T20 and T1144. TLT35 contains two active components, T20 and T1144. Both target gp41 NHR while having distinct and complementally functional domains. T20 contains LBD and HBD without PBD, whereas T1144 contains HBD and PBD without LBD. The T20 components target lipid membrane and the gp41 NHR groove, and the T1144 components target the gp41 NHR groove and deep pocket. Therefore, by linking T20 and T1144, TLT35 may simultaneously interact with gp41 NHR groove, deep pocket, and lipid membrane to prevent fusion core formation. The 35-mer linker may provide the space for TLT35 interaction with NHR and lipid membrane, as demonstrated in a series of four models (Fig. 6). The two components, T20 and T1144, in TLT35 may not be equally effective, as T1144 binds more strongly to NHR than T20. Consequently, some interaction models may dominate others. In the most sensitive HIV-1 strains, our results showed that the activity of TLT35 was close to that of T1144, suggesting that Model III may be the dominant binding model. Otherwise, in some drug-resistant viruses, especially T1144-insensitive viruses, Model IV might be dominant to overcome the weakness of T1144.

Although they are highly structured and very stable in 6-HB, either NHR- or CHR-peptide alone is usually unstructured or prone to aggregate in physiological condition. Accumulated evidence has shown that chemically modified CHR-peptides with stabilized α-helix conformation exhibit improved anti-HIV-1 potency because the modified CHR-peptides can form more stable 6-HB with the NHR-peptide or are more resistant to proteolysis (33). By linking multiple copies of NHR and CHR peptide sequences, chimera peptides may bury the hydrophobic surface of the peptides, thus preventing them from aggregation while allowing them at the same time to form a structure to increase the solubility and stability of fusion inhibitors (17). Chimera fusion inhibitors also have a suitable size for production as recombinant protein, thus potentially overcoming the high cost of synthesized peptide fusion inhibitors. As more fusion inhibitors are developed, they contain increasingly distinct functional domains and target multiple sites of gp41 and lipid membrane to prevent fusion core formation. As a result, interactions may occur among these fusion inhibitors of differing origins. Modifications of functional domains could provide useful sources for future chimera fusion inhibitor design.

TLT35 is stable in PBMC culture and is more resistant to proteolysis than T20 and/or T1144. High stability, which suggests longer half-life, means that the drug can be used with lower dosage and less frequent administration. The production of a recombinant protein is easier to scale-up than that of a synthetic peptide, suggesting that the production cost for TLT35 may be much lower than the costs associated with T20, which would in turn increase affordability for HIV-1/AIDS patients. Low nanomolar activity against various HIV-1 strains, including T20-resistant strains, also suggests that TLT35 is suitable for clinical trials for patients who fail to respond to T20 treatment. Application of TLT35 may also delay the emergence of drug-resistant strains. Overall, the high antiretroviral profile suggests that TLT35 is a promising candidate for development into a next generation fusion inhibitor with potential as either an anti-HIV-1 microbicide for prevention of HIV-1 sexual transmission or a novel anti-HIV-1 therapeutic for treatment of HIV-1 infection.

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