Design and Evaluation of Sifuvirtide, a Novel HIV-1 Fusion Inhibitor*

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Enfuvirtide (T20) is the first and only HIV-1 fusion inhibitor approved for clinical use, but it can easily induce drug resistance limiting its practical application. A novel anti-HIV peptide, termed sifuvirtide, was designed based on the three-dimensional structure of the HIV-1 gp41 fusogenic core conformation. Here we report its in vitro anti-HIV potency, its mechanism of action, as well as the results from Phase Ia clinical studies. We demonstrated that sifuvirtide inhibited HIV-1-mediated cell-cell fusion in a dose-dependent manner and exhibited high potency against infections by a wide range of primary and laboratory-adapted HIV-1 isolates from multiple genotypes with R5 or X4 phenotypes. Notably, sifuvirtide was also highly effective against T20-resistant strains. Unlike T20, sifuvirtide could efficiently block six-helix bundle formation in a dominant negative fashion. These results suggest that sifuvirtide has a different mechanism of action from that of T20. Phase Ia clinical studies of sifuvirtide (FS0101) in 60 healthy individuals demonstrated good safety, tolerability, and pharmacokinetic profiles. A single dose regimen (5, 10, 20, 30, and 40 mg) by subcutaneous injection once daily at abdominal sites was well tolerated without serious adverse events. Pharmacokinetic studies of single and multiple administration of sifuvirtide showed that its decay half-lives were 20.0 ± 8.6 h and 26.0 ± 7.9 h, respectively. In summary, sifuvirtide has potential to become an ideal fusion inhibitor for treatment of HIV/AIDS patients, including those with HIV-1 strains resistant to T20.

Currently, over 33 millions people worldwide are living with human immunodeficiency virus (HIV),§ and around 20 million people have died from AIDS (www.unaids.org). Due to lack of effective vaccines, development of novel anti-HIV drugs is critical to save lives of people with HIV infection. So far, 31 anti-HIV drugs and combinations have been approved for clinical use, and most of them belong to reverse transcriptase inhibitors and protease inhibitors (www.fda.gov). Application of these drugs in various combinations, known as highly active antiretroviral therapy, has significantly reduced the morbidity and mortality of HIV/AIDS (1). However, more and more HIV/AIDS patients have failed to respond to highly active antiretroviral therapy regimens because of the emergence of variants resistant to the current treatment regimens (2, 3). Therefore, the development of new classes of anti-HIV drugs is urgently needed.

The envelope glycoprotein of HIV-1 that mediates the attachment and fusion of the viral envelope and the cellular membrane represents a major target for the development of novel antiretroviral therapeutics (4, 5). HIV-1 envelope glycoprotein is a trimer, each of which consists of two non-covalently associated subunits gp120 and gp41 (6, 7). Membrane fusion is triggered by the binding of gp120 on the virus initially with the CD4 molecule and subsequently with a chemokine receptor (CCR5 or CXCR4) on the surface of the target cell (8, 9). These interactions result in a cascade of conformational changes that lead to the formation of a pre-hairpin intermediate of gp41 in which the hydrophobic N-terminal heptad repeat (NHR) are exposed and allows the fusion peptides to insert into the target cell membrane (5, 7). This transient gp41 intermediate then refolds into a stabilized trimer of hairpins, also called six-helix bundle structure (6-HB), which brings the close proximity of the viral envelope and the target cell membrane and results in the completion of the fusion process. Crystallographic and NMR studies show that the core of the hairpin consists of a parallel trimeric coiled-coil of NHR with the C-terminal heptad repeat (CHR) wrapped on the outside in an anti-parallel fashion (10–12). In addition, this structure feature has been found in the fusion-mediating subunits of other enveloped viral glycoproteins such as the S2 subunit of SARS corona virus, the GP2 subunits of Ebola virus, the HA2 subunit of influenza virus, the F1 subunits of the paramyxovirus simian virus 5 and human T cell lymphotropic virus type 1, and the TM subunits of retroviruses simian immunodeficiency virus, HIV-2, Moloney murine leukemia virus, and human respiratory syncytial virus-1 (13).
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Inhibitors capable of disrupting the fusion process hold great promise for clinical antiviral therapeutics. Extensive efforts are being made to develop novel peptide-based as well as small molecule inhibitors targeting the discrete regions of the prefusion intermediate during the fusion step (14, 15). In general, two major classes of such inhibitors have been described. The first one targets the NHR trimeric coiled-coil either by competitive binding with viral intrinsic CHR or by forming a heterotrimeric NHR with mutated NHR whereby disrupting the subsequent binding with native CHR (15–17). For examples, the peptides derived from the CHR such as T20 and its derivative T-1249 demonstrated potent anti-HIV activity in infected individuals, and T20 has been approved for clinical use. A series of mutated NHR peptides highlighted by N36mut(e.g.), where all residues at positions e and g of the helical wheel of the NHR trimeric coiled-coil were mutated, also demonstrated improved anti-HIV activity (16). Small molecule inhibitors targeting the deep pocket formed by the adjacent NHR monomers are also being investigated (15, 17). The second class targets the CHR of gp41. A representative of this class is a recombinant 5-helix protein in which only one of the N-helices in the presumably 6-HB structure is exposed (18). In addition, a recombinant NHR trimeric coiled-coil formed through artificial disulfide bridges such as N36mtGG and N36mtGG–N13 has also been shown to potently inhibit HIV fusion in \textit{in vitro} assays (19). Furthermore, it has recently been demonstrated that different classes of inhibitors targeting the NHR and CHR, and other classes of antiretroviral drugs, can be synergistic in suppressing viral replications both \textit{in vitro} and \textit{in vivo} (20).

Based on the three-dimensional structural information of HIV-1 gp41, we have designed a novel anti-HIV peptide, designated sifuvirtide (SFT) (see Fig. 1). This peptide inhibitor has been engineered with different sequence and/or location in the NHR versus the native CHR peptide. However, of these 36 residues, sifuvirtide is different from C34 in 16 residues and from T20 in 22 residues. Here, we report its \textit{in vitro} anti-HIV-1 potency, its mechanism of action, and the results from Phase Ia clinical studies.

**EXPERIMENTAL PROCEDURES**

**Trial Participants, Informed Consent, and Institutional Review Board**—A clinical Phase Ia study (FS0101) was designed to evaluate sifuvirtide for its safety, tolerability, and pharmacokinetic profiles in 60 healthy individuals. Subjects were healthy Chinese male volunteers aged 20–40 years with body weight of >45 kg and body mass index of 20.0–27.0 kg/m². Furthermore, subjects were in good healthy condition and were not addicted to smoking or drinking. Written informed consent was obtained from all subjects. Before the study began, the protocol and the informed-consent provisions were reviewed and approved by the independent institutional review board at the study site.

**Peptide Synthesis**—Peptides (sifuvirtide, C34, T20, and N36) were synthesized on a solid phase support, purified by reversed-phase high pressure liquid chromatography, and verified for purity by mass spectrometry and amino acid composition. Concentrations of the peptides were determined by UV absorbance and a theoretically calculated molar-extinction coefficient $\epsilon$ (280 nm) of 5500 M$^{-1}$.cm$^{-1}$ and 1490 M$^{-1}$.cm$^{-1}$ based on the number of tryptophan and tyrosine residues (all the peptides tested contain Trp and/or Tyr), respectively.

**CD Spectroscopy**—CD spectroscopy was performed as previously described (21). Briefly, an N-peptide (N36) was incubated with the equal molar concentration of C-peptide (C34 or T20) or sifuvirtide at 37 °C for 30 min. The final peptide concentration was 10 μM in 50 mM sodium phosphate and 150 mM NaCl, pH 7.2. The isolated N- and C-peptides as well as sifuvirtide were also tested. CD spectra of these individual peptides and peptide mixtures were acquired on a Jasco spectropolarimeter (Model J-715, Jasco Inc., Japan) at room temperature using a 5.0 nm band with, 0.1 nm resolution, 0.1-cm path length, 4.0-s response time, and a 50 nm/min scanning speed. The spectra were corrected by subtraction of a blank corresponding to the solvent. The $\alpha$-helical content was calculated from the CD signal by dividing the mean residue ellipticity at 222 nm by the value expected for 100% helix formation (−33,000 degrees cm$^2$ dmol$^{-1}$) according to previous studies (22, 23). Thermal denaturation was monitored at 222 nm by applying a thermal gradient of 2 °C/min in the range of 4–98 °C. To determine the reversibility, the peptide mixtures were cooled to 4 °C and kept in the CD chamber at 4 °C for 30 min, followed by monitoring of thermal denaturation as described above. The melting curve was smoothed, and the midpoint of the thermal unfolding transition ($T_m$) values was calculated using Jasco software utilities.

**Native-PAGE**—Native-PAGE was carried out to determine the 6-HB formation between the N- and C-peptides as described previously (24). Briefly, an N-peptide (N36) was mixed with a C-peptide (C34 or T20) or sifuvirtide at a final concentration of 40 μM and incubated at 37 °C for 30 min. The mixture was loaded onto 10 × 1.0-cm precast 18% Tris-glycine gels (Invitrogen) at 25 μl/well with an equal volume of Tris-glycine native sample buffer (Invitrogen). 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 FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA).

**Inhibition of Sifuvirtide on the 6-HB Formation**—Inhibitory activity of sifuvirtide on the 6-HB formation was measured by a modified ELISA-based method as previously described (25). Briefly, a 96-well polystyrene plate (Costar, Corning Inc., Corning, NY) was coated with a 6-HB-specific monoclonal antibody NC-1 IgG (4 μg/ml in 0.1 M Tris, pH 8.8). A tested peptide (sifuvirtide, C34 or T20) at graded concentrations was mixed with C34–biotin (0.25 μM) and incubated with N36 (0.25 μM) at room temperature for 30 min. The mixture was then added to the NC-1-coated plate, followed by incubation at room temperature for 30 min and washing with a washing buffer (PBS containing 0.1% Tween 20) three times. Then streptavidin-labeled horseradish peroxidase (Invitrogen) and the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma) were added sequentially. Absorbance at 450 nm ($A_{450}$) was measured using an ELISA reader (Ultra 384, Tecan, Research Triangle Park, NC). The
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percent inhibition by the peptides and the IC$_{50}$ values were calculated as previously described (26).

Peptide-Lipid Binding Measured by Isothermal Titration Calorimetry—Large unilamellar vesicles (LUVs) of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposome were prepared as described (27). Briefly, 20 mg of POPC stock solution was dried under a stream of nitrogen and stored under vacuum overnight to completely remove trace amounts of organic solvent. The dried lipid film was suspended by vortexing in 2 ml of PBS buffer. The resultant multilamellar vesicle suspension was freeze-thawed for 5 cycles and then successively extruded through two stacked polycarbonate filters (0.1 μm) 13 times using an Avanti miniexxtruder. Binding of sifuvirtide, C34, or T20 to POPC LUVs was measured using a high sensitivity isothermal titration calorimetry instrument (MicroCal, Northampton, MA) as previously described (27). Solutions were degassed under vacuum prior to use. LUVs of POPC were injected into the chamber containing a peptide solution or buffer only (as a control). The heats generated in control experiments by injecting lipid vesicles into buffer without a peptide were subtracted from the heats produced in the corresponding peptide-lipid binding experiments. Data acquisition and analyses were performed using MicroCal Origin software (version 7.0).

Peptide-Lipid Binding Measured by Fluorescence Spectroscopy—Fluorescence spectra of the peptides sifuvirtide, C34, or T20 (10 μM) presented in PBS and POPC LUVs (2 mM), respectively, were obtained on a Hitachi fluorescence spectrophotometer, with an excitation wavelength of 295 nm, and emission was scanned from 300 to 450 nm at a scan rate of 10 nm/s. Spectra were baseline-corrected by subtracting blank spectra of the corresponding solutions without peptide.

Cell-Cell Fusion Assay—A dye transfer assay was used for detection of HIV-1-mediated cell-cell fusion as previously described (28). In brief, H9/HIV-1$_{lu}$-infected cells were labeled with a fluorescent reagent, Calcein-AM (Molecular Probes, Inc., Eugene, OR), and then incubated with MT-2 cells (ratio = 1:5) in 96-well plates at 37 °C for 2 h in the presence or absence of peptides. The fused and unfused calcein-labeled HIV-1-infected cells were counted under an inverted fluorescence microscope (Zeiss, Germany) with an eyepiece micrometer disc. The percent inhibition of cell-cell fusion and the IC$_{50}$ values were calculated as described before (28).

Measurement of Anti-HIV-1 Activity of SFT and T20—The inhibitory activity of sifuvirtide on infection by various T20-resistant virus isolates and laboratory-adapted HIV-1 strain (HIV-1$_{IIIb}$) was determined as previously described (28). In brief, 1 × 10$^5$ MT-2 cells were infected with HIV-1 isolates at 100 TCID$_{50}$ (50% tissue culture-infective dose) in 200 μl of RPMI 1640 medium containing 10% fetal bovine serum in the presence or absence of sifuvirtide at graded concentrations overnight. Then the culture supernatants were collected, and lysine residues were introduced into the sifuvirtide to favor the formation of ion pairs (salt bridges) at the i and i+4 positions of the helical conformation. Glu$^{119}$ was substituted by threonine to cover the hydrophobic pocket. Furthermore, a serine was added to the N terminus of the peptide, because addition of this residue to the N-terminal of an α-helix is expected to increase its stability. After a series of the above-mentioned alterations, the resulting peptide sifuvirtide is composed of 36 residues in length and shares some sequence and structure featur-
tures with the native CHR peptide. Of these 36 residues, sifuvirtide is different from C34 in 16 residues and from T20 in 22 residues (Fig. 1).

**Biophysical Characterization of SFT**—To study the secondary structure of sifuvirtide, we have measured its CD spectra in the presence or absence of peptide N36. Under the experimental conditions, the CD spectra of sifuvirtide, C34, N36, and T20 did not show a minimum at 222 nm (Fig. 2A), suggesting that these peptides alone have minimum α-helical conformation. The CD spectrum for sifuvirtide is similar to that of C34 and T20 and is typical of peptide with a random coil conformation (29). In contrast, the CD spectra of sifuvirtide in the presence of N36 display a double minimum at 208 and 222 nm, suggesting that the interaction between sifuvirtide and N36 induces the formation of secondary α-helical structure. Similarly, mixing of peptides C34 and N36 also led to the formation of α-helical complexes manifested by an almost overlapping CD profile with that of sifuvirtide (Fig. 2A). Quantization of the CD data indicated a helical content of 93% for sifuvirtide and 85% for C34 peptide in the presence of N36. T20, on the other hand, does not form α-helical complexes with N36 peptide demonstrated by the lack of minimum at 208 and 222 nm. The result indicated that sifuvirtide binds to N36 in a comparable manner of C34 by forming a typical α-helix, which is in great contrast to that of T20.

We then monitored the changes in CD spectra of sifuvirtide/N36 complexes over increasing temperatures from 4 to 98 °C to study the binding affinity of sifuvirtide to N36. Fig. 2B shows the CD thermal denaturation curve of sifuvirtide/N36 in comparison with that of C34/N36 complexes. The sifuvirtide/N36 complexes are found to have a significantly higher level of α-helical content and be more heat-stable than that of C34/N36. The melting temperature (T_m) of sifuvirtide/N36 complexes was ~72 °C, whereas that of C34/N36 was ~62 °C (Fig. 2B, inset). The increment of 10 °C suggests higher affinity between sifuvirtide and N36 and higher stability of sifuvirtide/N36 complex compared with C34/N36. Such biophysical characters of sifuvirtide are consistent with our initial aim of peptide design, which is to increase the stability, α-helical propensity, as well as binding capacity to the N-helices. However, T20 cannot form α-helical structure with N36 under our experimental conditions, consistent with previous reports (30).

**Unlike T20, SFT Is Able to Form 6-Helix Bundle with N36**—We previously developed a native-PAGE-based method to visualize the 6-HB formed by N36/C34 and used it to characterize the antiviral activity of HIV-1 fusion inhibitors (24,30). In this study, native-PAGE was used to study the interaction between sifuvirtide and N36 and its potential in forming the 6-helix bundle. As shown in Fig. 3, N36 (lane 1) exhibited no band, because it carries net positive charges and may migrate up and off the gel. C34 (lane 2), sifuvirtide (lane 3), and T20 (lane 4) each displayed a band in the lower part of the gel. Like C34, sifuvirtide was able to form 6-HB with N36 reflected by the up-shift of a peptide band comparing to sifuvirtide or C34 alone. In contrast, no such changes were found for T20 after mixing with N36, suggesting that T20 and N36 do not form similar complexes like that of sifuvirtide or C34. These findings are consistent with our observations from CD studies (see above).
To further analyze the interaction between sifuvirtide with N36, we studied whether sifuvirtide can inhibit the formation of 6-HB between C34-biotin and N36 in solution by our antibody-based ELISA method (25). Both sifuvirtide and C34 were able to disrupt the formation of 6-HB in a dose-dependant manner, whereas T20 failed to do so (Fig. 4). In addition, sifuvirtide demonstrates significant higher potency in disrupting the formation of 6-HB than C34, manifested by its IC_{50} value of 0.12 μM compared with 0.31 μM of C34 (Fig. 4).

Unlike T20, SFT Cannot Interact with Lipid Membrane—Previous studies suggest that the CHR helices located in the outer layer of the 6-HB may interact with the lipid membranes and participate in the formation of fusion pore (31). Using the POPC LUV liposome system in an isothermal titration calorimetry assay, we measured the heats generated from the interaction of a peptide (sifuvirtide, C34, or T20) with lipid vesicles. As shown in Fig. 5A (upper panel), large heat release was detected when POPC LUVs was added into a solution containing T20. The calculated binding constant of T20 to POPC LUVs was \(-5 \times 10^4\) M\(^{-1}\). However, the bindings of sifuvirtide and C34 to POPC LUVs were too weak to calculate the binding constant (Fig. 5A, lower panel). Further, we used fluorescence spectroscopy to determine the interaction of the peptides with lipid bilayers. Because all three peptides contain tryptophan residues, the tryptophan fluorescence emission spectra of the peptides may change if they interact with the lipid bilayers. Significant blue shift of the fluorescence spectra was observed when the peptide T20 was presented in the POPC LUVs (Fig. 5B, lower panel), compared with their presence in PBS (Fig. 5B, upper panel), whereas sifuvirtide and C34 did not show remarkable fluorescence spectra shift. These results suggest that, unlike T20, both sifuvirtide and C34 may not interact with the target cell membrane.

Potent Inhibition of SFT on HIV-1-mediated Cell-Cell Fusion and Infection—It is important to know whether the engineered peptide sifuvirtide has anti-HIV activity. First, we measured the inhibitory activity of sifuvirtide on HIV-1\textsubscript{IIIB}-mediated cell-cell fusion. As shown in Fig. 6A, sifuvirtide could inhibit the fusion with an IC_{50} of 3.60 nm. In comparison, T20 inhibited the fusion with an IC_{50} of 21.39 nm. Thus, sifuvirtide is \(-6\)-fold more active than T20 in inhibiting fusion of viral and cellular membranes. Then, we assessed sifuvirtide for its inhibitory activity on HIV-1-mediated infection. Consistently, sifuvirtide was also more potent to inhibit HIV-1\textsubscript{IIIB}-mediated infection of MT-2 cells than T20 (Fig. 6B). We then tested sifuvirtide and T20 with a panel of primary HIV-1 isolates. As shown in Table 1, sifuvirtide was highly active in inhibiting primary HIV-1 isolates with distinct genotypes and phenotypes (subtypes A–F).

SFT Inhibits Replication of T20-resistant HIV Strains—Because there were significant differences in biophysical and anti-HIV activities between sifuvirtide and T20, we sought to determine whether sifuvirtide is able to inhibit replication of HIV-1 strains resistant to T20. Two T20-sensitive and five T20-resistant strains with well defined genetic mutations conferring the resistance were used in our experiments (32). We found that both sifuvirtide and T20 were active against T20-sensitive strains, but sifuvirtide was more potent than T20, consistent with our above observation using both laboratory-adapted and primary HIV-1 viruses. More importantly, sifuvirtide showed strong inhibitory activities against all T20-resistant strains with IC_{50} ranging from 2.68 to 47.78 nm, whereas T20 failed to do so even with concentration at least 40- to 700-fold higher (Table 2). These results suggest that sifuvirtide can be used as an alternative fusion inhibitor for treatment of patients with HIV-1/AIDS, in particular those infected by T20-resistant variants.

Safety and Pharmacokinetic Studies of SFT—No subject was withdrawn from Phase Ia studies due to adverse events or toxicity. The possible drug-related side effects were observed in 7 subjects in the safety study of single dose of sifuvirtide, including injection site reaction in 1 subject (40 mg group), elevations of alanine aminotransferase in 2 subjects (5- and 20-mg group), elevations of IgD in 3 subjects (10-mg group), and elevation of IgE in 1 subject (30-mg group). All the events were mild and recovered without action. In a pharmacokinetic study of a single dose of sifuvirtide, safety was also investigated. Two subjects had possible drug-related events, skin rash in one subject, and elevation of IgD in one subject. Fig. 7A shows the pharmacokinetics profile of single dose administration of 10–30 mg of sifu-
The pharmacokinetics process showed a linear relationship in principle, $t_{1/2}$ was $20.0 \pm 8.6$ h (Table 3), and valley concentration was higher than anti-HIV concentration in vitro.

Safety and pharmacokinetics studies of multiple doses of sifuvirtide were further conducted in additional 12 volunteers with repeated subcutaneous injection of 30 mg of sifuvirtide once daily for 7 days. One subject had an injection site reaction. Two had an elevation of eosinophilia. One had a transient elevation of alanine aminotransferase, $\gamma$-glutamyltranspeptidase, and lactic acid dehydrogenase. These adverse events were considered possibly related to the drug. The mean steady concentration of sifuvirtide was $151 \pm 34$ ng/ml, and $t_{1/2}$ was $26 \pm 7.9$ h (Table 4), and these are consistent with those of single dose administration. Therefore, this dose in consecutive administration was safe and had a satisfactory profile of pharmacokinetics as well (Fig. 7B).

**DISCUSSION**

In the present study, we have designed a novel anti-HIV peptide based on the structural information and molecular modeling analysis of HIV-1 gp41. The peptide sifuvirtide was rationally engineered to improve its pharmaceutical properties. Biophysical analyses demonstrated that sifuvirtide may have a different mechanism of action from T20. Unlike T20, sifuvirtide could form stable 6-HB structure with NHR-derived peptide N36 and had more potent inhibitory activity against HIV-1 isolates with distinct genotypes and phenotypes. In particular, sifuvirtide was also highly effective against T20-resistant strains. Our clinical Phase Ia studies demonstrated that sifuvirtide has good safety, tolerability, and pharmacokinetic profiles, suggesting its potential as an effective fusion inhibitor for treatment of HIV/AIDS.

Previous studies indicate that peptides derived from the NHR and CHR of HIV-1 gp41 have potent antiviral activity (15, 17). For example, two CHR-derived peptides, C34 and T20, are most active prototypic inhibitors on HIV-1-mediated cell-cell fusion and infection. Although these two peptides are largely overlapped by their NHR-binding domain, C34 contains the pocket-binding domain at its N terminus that is essential for its anti-HIV activity (27, 33, 34). In comparison, T20 lacks the pocket-binding domain, but it has a tryptophan-rich region at its C terminus, which is critical for the anti-HIV activity through the interaction with the lipid on the cellular membrane (27, 30). The peptide C34 has more potent antiviral activity, but it may not be a good drug candidate due to its low solubility as...
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TABLE 1
Potent inhibition of SFT on primary HIV-1 isolates

| Virus Phenoype | IC_{50} SFT (%) | IC_{90} SFT (%) | T20 IC_{50} | IC_{90} |
|----------------|-----------------|-----------------|-------------|--------|
| 94UG103 (clade A, X4R5) | 3.94 ± 1.29 | 7.43 ± 2.16 | 83.84 ± 12.09 | 150.69 ± 8.80 |
| 92US657 (clade B, R5) | 27.78 ± 8.66 | 211.78 ± 83.96 | 41.45 ± 8.76 | 177.33 ± 15.95 |
| 93IN101 (clade C, R5) | 34.31 ± 3.21 | 90.64 ± 6.50 | 47.21 ± 4.73 | 129.69 ± 16.76 |
| 92UG001 (clade D, X4R5) | 11.92 ± 3.01 | 35.68 ± 6.59 | 185.10 ± 23.34 | 501.40 ± 70.78 |
| 92TH009 (clade E, R5) | 209.77 ± 9.91 | 618.65 ± 39.04 | 221.44 ± 58.31 | 724.35 ± 56.64 |
| 93BR020 (clade F, X4R5) | 12.46 ± 6.76 | 71.64 ± 28.54 | 73.68 ± 8.57 | 204.26 ± 28.01 |

TABLE 2
Potent inhibition of SFT on T20-resistant viruses

| Virus Phenotype | IC_{50} SFT (%) | mean ± S.D., nM |
|-----------------|-----------------|-----------------|
| NL4-3 (parental) | S | 22.04 ± 1.19 | 12.56 ± 1.18 |
| NL4-3 (N42S) | S | 26.95 ± 12.98 | 2.68 ± 0.87 |
| NL4-3 (V38A) | R | >2000 | 3.44 ± 0.23 |
| NL4-3 (V38A/N42D) | R | >2000 | 47.78 ± 4.70 |
| NL4-3 (V38A/N42T) | R | >2000 | 30.42 ± 4.36 |
| NL4-3 (V38E/N42S) | R | >2000 | 43.47 ± 3.36 |
| NL4-3 (N42T/N43K) | R | >2000 | 37.79 ± 15.55 |

* Sensitive (S) or resistant (R) to T20.

TABLE 3
Comparison of pharmacokinetic parameters after subcutaneous injection of different doses of SFT in healthy volunteers (3 × 3 cross-over design)

| Parameter | Unit | Multiple dose study | Single dose study |
|-----------|------|---------------------|------------------|
| AUC(0–24h) | ng h ml^{-1} | 3264 ± 793 | 3615 ± 825 |
| AUC(0–72h) | ng h ml^{-1} | 3512 ± 2764 |
| AUC(0–∞) | ng h ml^{-1} | 3512 ± 2764 |
| AUC(72–∞) | ng h ml^{-1} | 3512 ± 2764 |
| AUC(72–∞) | % | 3512 ± 2764 |
| MRT | h | 31.5 ± 10.8 |
| k_{d} | h^{-1} | 0.0283 ± 0.0112 |
| C_{max} | ng ml^{-1} | 97 ± 62 |
| t_{1/2} | h | 3.18 ± 1.0 |
| T_{max} | h | 4.0 ± 0.0 |

* p < 0.05 versus subcutaneous 10-mg group.

antiviral activity and a longer half-life than T20, but its clinical development has been suspended due to the problem of drug formulation. Therefore, new peptide-based HIV-1 fusion inhibitors that overcome the above problems are promising drug candidates.

Several approaches have been taken to engineer the peptides for improving their pharmacokinetic profiles and anti-HIV activity. For example, introducing ion pair interactions or salt bridges has been proven to increase the helicity of the peptides and stabilize the coiled-coils (38–40). Otaka et al. (35) synthesized a C34 mutant, SC34EK, in which eight i and i + 4 Glu-Lys ion pairs were introduced to form intrahelical salt bridges that enhance the solubility and α-helicity of the peptide. As expected, SC34EK had ~3-fold higher anti-HIV activity than the parent C34. In our design, we introduced several salt bridges into the sifuvirtide to favor its solubility, α-helicity, and thereby its durability and antiviral potency (Fig. 1). The 6-HB formed by sifuvirtide and its counterpart NHR peptide (N36) shows 93% helicity with a T_m value of 72 °C as measured by CD spectroscopy. In comparison, the 6-HB formed by N36-C34, which is considered as a core structure of the fusion-active gp41, has a T_m of 62 °C. Therefore, sifuvirtide might target the

FIGURE 7. Pharmacokinetics of SFT in healthy volunteers determined in a open, randomized, non-controlled clinical Phase Ia trial. A, pharmacokinetics of SFT single dose administration in 12 healthy volunteers (3 groups, 4 volunteers per group). B, pharmacokinetics of SFT successive administration in 12 healthy volunteers.

compared with T20 (35). T-1249 is a second generation fusion inhibitor designed based on the HIV-1, HIV-2, and simian immunodeficiency virus CHR sequences (36, 37). It has greater
Sifuvirtide demonstrated high inhibitory activities against a wide variety of primary isolates with different genotypes and phenotypes. Although sifuvirtide is designed on the basis of the gp41 of HIV-1 subtype E, it also shows strong inhibitory activity on non-subtype E viruses, such as subtype B as shown in our study. It suggests that the target of sifuvirtide is broad enough to permit alternatives in gp41 amino acids. Another finding from our study shows that sifuvirtide is 6- to 20-fold more effective against X4R5 viruses than T20. It is known that R5 occurred in most patients newly infected by HIV-1, along with the progression of HIV, tropism presents by emerging of dual-mixed (X4R5) or pure X4-tropic virus, which account for 50% of the infected population (8, 41). Therefore, sifuvirtide should keep active for a long time in clinical use.

T20, the first peptidic HIV-1 entry inhibitor approved for the treatment of HIV-1 in infected patients (4), has many advantages: it acts outside cells and targets the protein of virus, rather than host cell. Therefore, it is expected to have low drug-related toxicity, compared with those acting inside cells or targeting cellular proteins. However, it has several weaknesses. Its shorter half-life (3.8 h) requires more frequent subcutaneous injections. High dosage (90 mg) and twice daily usage of T20 may result in the injection site reactions and financial burden for HIV/AIDS patients. In contrast, sifuvirtide improves in both aspects. Higher potency and longer half-life of sifuvirtide in humans (26 h) enable sifuvirtide to be given with less clinical dosage and less frequency of injections, which minimizes the injection site reactions and reduces financial burden of the treated patients. Although T20 has a pivotal role in optimizing antiretroviral drug combinations, it also has the problem of drug resistance (4). Sifuvirtide is highly effective against T20-resistant strains (Table 2). Therefore, sifuvirtide may be used to treat patients infected with T20-resistant HIV-1. We believe that sifuvirtide is an ideal fusion inhibitor with improved potency, half-life, and resistance profile that can be used for the treatment of HIV/AIDS patients, including those who are infected by HIV-1 strains resistant to T20.

Development of potent anti-HIV inhibitors are greatly needed in treating infected patients. New classes of anti-HIV drugs, such as integrase inhibitors and inhibitors against coreceptors CCR5, have showed great promise in suppressing HIV replication in vivo (4, 5, 42). These novel drugs have demonstrated in vitro synergism with other classes of antiretrovirals, thus offering the rationale for combinational use in treating infected patients. In addition, many efforts have also been made to develop novel candidates against additional viral targets such as viral fusion intermediate, viral assembly, and maturation. In line with developing novel candidate drugs against previously validated viral target such as viral fusion intermediate, sifuvirtide was designed and tested both in vitro and in vivo. Based on our results so far, we believe that sifuvirtide has unique features that will complement existing class of antiretrovirals. In particular, its potent activities against T20-resistant strains suggest that sifuvirtide will be able to play a significant role in the patients who have developed T20-resistant mutations. The in vivo efficacy of sifuvirtide in suppressing HIV-1 replication in infected patients is being evaluated in Phase II clinical trails.

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