CD4-anchoring HIV-1 Fusion Inhibitor with Enhanced Potency and in Vivo Stability*

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In this study, we describe a novel CD4-targeting bifunctional human immunodeficiency virus (HIV-1) fusion inhibitor (CD4-BFFI) that blocks HIV-1 entry by inhibiting both HIV-1 attachment and fusion and is highly potent against both R5 and X4 HIV-1 viruses in various antiviral assays, including peripheral blood mononuclear cell (PBMC) infection assays. Previously, we have reported a CCR5 antibody-based bifunctional HIV-1 fusion inhibitor (BFFI) that was highly active in blocking R5 HIV-1 infection but was ineffective against X4 viruses infecting human PBMCs (Kopetzki, E., Jekle, A., Ji, C., Rao, E., Zhang, J., Fischer, S., Cammack, N., Sankuratri, S., and Heilek, G. (2008) Virology J. 372, 56–65). CD4-BFFI, which consists of two HIV-1 fusion inhibitor (FI) T-651 variant peptides recombinantly fused to the C-terminus of a humanized anti-CD4 monoclonal antibody, has demonstrated more than 100-fold greater antiviral activity than T-651 variant or the parental CD4 monoclonal antibody. Mechanistic studies revealed that CD4-BFFI primarily blocks the HIV-1 cell-fusion step through its FI peptide moieties. The enhanced antiviral activity of CD4-BFFI is most likely due to avid binding of the bivalent FI peptides as well as the increased local concentration of CD4-BFFI via attachment to the target cell surface receptor CD4. In vivo pharmacokinetic studies demonstrated that CD4-BFFI was stable in monkey blood, and a dose of 10 mg/kg maintained serum concentrations greater than 2,000-fold over the IC50 value for 7 days postdosing. This novel bifunctional inhibitor with improved potency and favorable pharmacokinetic properties may offer a novel approach for HIV-1 therapy.

Human immunodeficiency virus, type 1 (HIV-1) enters the host cell through viral envelope-cell membrane fusion. HIV-1 recognizes its host cells by binding to the cell surface receptor CD4 and coreceptors via its envelope protein gp120. gp120 is a globular glycoprotein which is noncovalently associated with the transmembrane protein gp41. Upon binding to CD4, gp120 undergoes conformational changes allowing it to interact with one of two major coreceptors: CCR5 or CXCR4. Although the majority of primary HIV-1 strains during early phases of infection use CCR5 as coreceptor (termed R5 virus), some viruses use CXCR4 (termed X4 virus) or both CCR5 and CXCR4 (termed R5X4 virus or dual-tropic viruses) (1).

Both gp120 and gp41 exist as trimers on the HIV-1 particle surface. HIV-1 gp41 contains three domains: an extracellular, a transmembrane, and a cytoplasmic domain. The extracellular domain contains an amino-terminal hydrophobic fusion peptide and two heptad repeats (HR). Binding of gp120 to the coreceptor triggers structural rearrangement in gp41, leading to virus-host cell fusion. The fusion process begins with the insertion of the gp41 fusion peptide into the host cell membrane, forming a bridge between virus and cell. The COOH-terminal HR (HR2) domains then associate with the NH2-terminal HR (HR1) domains in an anti-parallel manner. This HR1-HR2 interaction results in the formation of a six-helix bundle (three HR2 helices packed on the outer surface of the trimeric HR1 helices) (2, 3). This six-helix bundle formation brings the viral and cell membranes to close proximity, thus resulting in virus-cell fusion and the entry of the viral core into the host cell cytoplasm.

The highly active anti-retroviral therapy regime, a combination therapy comprising three or more anti-HIV-1 agents, targeting one or more HIV-1 or host proteins, is the current standard of care for HIV-1/AIDS patients. The majority of the HIV-1 drugs on the market target HIV-1 enzymes, such as the reverse transcriptase, protease, and integrase. The emergence of drug resistance to the currently available treatment options necessitates the development of new classes of anti-HIV-1 drugs (4). One of the steps in the viral life cycle for therapeutic intervention is the viral entry process (5–7). Drugs that target the HIV-1 entry steps have shown potent antiviral effects in preclinical and clinical studies. The first HIV-1 entry inhibitor on the market, enfuvirtide (ENF, T-20), is an HR2-derived peptide inhibitor that targets the six-helix bundle formation (6). Maraviroc, a second HIV-1 entry inhibitor approved in 2007, is a CCR5 antagonist. However, maraviroc only blocks R5 HIV-1 entry and not X4 or dual-tropic viruses (8).
CD4-anchoring HIV-1 Entry Inhibitor

Despite its clinically proven efficacy, the use of ENF is limited by its twice daily dosing regimen (due to rapid elimination of the peptide from plasma), as well as the low genetic barrier to resistance and injection site reactions in some patients. Recently, we reported a novel strategy to enhance the in vivo exposure of HIV-1 fusion inhibitor (FI) while maintaining its antiviral potency against R5 viruses (9). This strategy used a CCR5 monoclonal antibody (mAb) as the scaffold for the fusion inhibitor peptide. This mAb-FI chimeric protein contains two HIV-1 entry inhibitors: a CCR5 mAb that blocks HIV-1 attachment to the CCR5 coreceptor and a FI that blocks the HIV-1-host cell fusion; thus, it was named bifunctional HIV-1 fusion inhibitor (BFFI). BFFI showed greater antiviral potency than either the FI peptide or the CCR5 mAb in in vitro antiviral assays. However, it failed to block the entry of X4 and R5X4 viruses into subsets of HIV-1 target cells expressing CXCR4 but not CCR5, suggesting that anchoring of this bifunctional molecule to the target cell through CCR5 is essential for X4 inhibitory activity (9).

Here, we describe a second generation BFFI molecule that is equally potent in inhibiting R5 and X4 HIV-1 entry. This new BFFI utilizes a CD4 mAb instead of a CCR5 mAb for anchoring to the target cell surface and thus was named CD4-BFFI, to differentiate it from the previously reported CCR5 mAb-based BFFI (CCR5-BFFI). The CD4 mAb used in the CD4-BFFI molecule was derived from the well studied humanized anti-human CD4 mAb TNX-355 (also called Hu5A8) (10, 11). TNX-355 had equal potency in inhibiting R5 and X4 viruses in in vitro assays. CD4-BFFI demonstrated higher potency than the fusion inhibitor T-651 variant (T651v) or the parental CD4 mAb alone. Moreover, CD4-BFFI is highly stable in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Reagents, Viruses, and Cell Lines—JC53BL (TZM-b1) cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent program. 293T human embryonic kidney cells and HEK293-EBNA cells were obtained from ATCC (Manassas, VA). MAGI-CCR5 cells were generated in house (9). Human PBMCs were obtained from AllCells (Emeryville, CA), stimulated for 1 day in PBMC medium (RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM minimum Eagle’s medium nonessential amino acids) supplemented with 2 μg/ml phytohemagglutinin (all from Invitrogen) and maintained in PBMC medium containing 5 units/ml human interleukin-2 (Roche Applied Science). NL4-3 was obtained from the National Institutes of Health AIDS Research and Reference Reagent program. The NH2-terminally acetylated COOH-terminally amidated fusion inhibitor peptides T-651v and T-651v mutant (T-651mut) were chemically synthesized in-house as described before (14).

Expression Plasmids—For the construction of the bifunctional human CD4 antibody-based fusion inhibitors, the variable domains (VH and VL) of the humanized human CD4-specific mAb (hu5A8, also called TNX-355) (15) were cloned and expressed as the human IgG1 isotype, which carries L234A and L235A double mutations (IgG1-LALA). This reconstructed anti-CD4 mAb was named 6314. Antibody light and heavy chain genes are expressed from two identical assembled expression units, including the genomic exon-intron structure of antibody genes. The antibody light and heavy chain expression cassettes are located on the same plasmid in a clockwise orientation. Expression of cognate antibody light and heavy chain is controlled by a shortened intron A-deleted immediate early enhancer and promoter from the human cytomegalovirus and the strong polyadenylation signal from bovine growth hormone. All expression plasmids also contain an origin of replication and a β-lactamase gene from the vector pUC18 for plasmid amplification in Escherichia coli and a neomycin resistance gene for the generation/selection of stably transfected CHO cell lines (Fig. 1A).

The structural gene of the light chain was assembled by fusing a chemically synthesized hu5A8 variable light chain cDNA at the 5′-end with a DNA segment encoding a murine immunoglobulin heavy chain signal sequence (containing an intron) and at the 3′-end with a DNA segment containing a splice donor site and a unique BamHI restriction site. The BamHI restriction site was used to fuse hu5A8 variable light chain to the human κ-light chain gene constant region, including a truncated human κ-light chain intron 2. The structural gene of the heavy chain was assembled by fusing a chemically synthesized hu5A8 variable heavy chain cDNA at the 5′-end with a DNA segment encoding a murine immunoglobulin heavy chain signal sequence (containing an intron) and at the 3′-end with a DNA segment containing a splice donor site and a unique Xhol restriction site. The Xhol restriction site was used to join the hu5A8 variable heavy chain with the genomic human yl-heavy chain gene constant region (containing a truncated human yl-heavy chain intron 2).

For the construction of various CD4-BFFI molecules, the DNA sequences encoding the entire GS peptide linker (GGGGSGGGGGSGGGGSG) and the various FI peptides were chemically synthesized and inserted into the expression plasmids through unique restriction sites. A unique HindIII restriction site was used to join the linker-FI at its 5′-end to the COOH terminus of the heavy chain of CD4 mAb 6314, and a unique NheI restriction site was used to join linker-FI at its 3′-end to the bovine growth hormone polyadenylation signal gene (Fig. 1A). The HIV-1 FI T-651v was derived from the gp41 ectodomain of HIV1B8 HIV-1 reference strain (BH8 isolate; positions 610–656; residue numbering is based on the envelope polypeptide gp160 precursor) (16). This HIV-1 gp41-derived peptide contains two potential N-linked glycosylation sites, one at the NH2 terminus (NMT) and the other within the peptide. To avoid N-glycosylation that may reduce the antiviral activity of T-651v, the potential N-glycosylation site (NYT) within the peptide is inactivated through a point mutation to change Asn to Gln in all BFFI molecules except in BFFI-G2. In CD4-BFFI-G0, the NH2-terminal N-glycosylation site is also eliminated by deletion. The CD4-BFFImut molecule contains the T-651v peptide that carries four additional amino acid mutations to eliminate its antiviral activity (Fig. 1B).

Expression and Purification of Antibodies—All antibodies were expressed by transient transfection of human embryonic kidney 293F cells using the FreeStyle™ 293 expression system.
according to the manufacturer's instructions (Invitrogen). Antibody-containing culture supernatants were filtered and purified by two chromatographic steps. Antibodies were captured by affinity chromatography using Protein A-Sepharose™ CL-4B (GE Healthcare) equilibrated with 0.1 M phosphate buffer, pH 7.0. Unbound proteins were washed out with equilibration buffer, and the antibodies were eluted with 0.1 M citrate buffer, pH 3.5, and then immediately neutralized to pH 6.0 with 1 M Tris-base. Size exclusion chromatography on Superdex 200™ (GE Healthcare) was used as a second purification step. Size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The eluted antibodies were concentrated with an Ultrafree®-CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at −80 °C.

Analytic Characterization of Antibodies—Analytical characterization of antibody proteins was performed as described before (9). N-Linked glycosylation of fusion inhibitor peptides was evaluated by SDS-PAGE and by comparison of wild type and PNGase F-treated BFFI samples. N-Linked carbohydrates were released by enzymatic treatment with 50 milliunits of PNGase F (Roche Molecular Biochemicals) per mg of protein at 37 °C for 12–24 h at a protein concentration of about 2 mg/ml.

HR1 Western Blot—The BFFI molecules were detected by the interaction of the fused FI peptide sequences (derived from the HR2 of HIV-1 gp41) with its natural viral interaction partner, the HR1 of HIV-1 gp41. BFFI molecules were resolved by SDS-PAGE and blotted to membrane, and the FI-containing heavy chains were visualized by probing with biotinylated T-2324 HR1 peptide: Biotin-QARQLSGLVQQQNLRARLAIAQOHLLQLTVGKQILARILVERLKDQ-NH₂ (COOH-terminally amidated). The blocked membranes were incubated with 0.5 μg/ml HR1 peptide T-2324 in 0.5% (w/v) Western Blocking Reagent (Roche Applied Science) at 4 °C with shaking overnight or at least 2 h at room temperature. The blocked membranes were incubated with 0.5 μg/ml HR1 peptide T-2324 in 0.5% (w/v) Western Blocking Reagent (Roche Applied Science) at 4 °C with shaking overnight or at least 2 h at room temperature. The blocked membranes were incubated with 0.5 μg/ml HR1 peptide T-2324 in 0.5% (w/v) Western Blocking Reagent (Roche Applied Science) at 4 °C with shaking overnight or at least 2 h at room temperature, and then stained with a streptavidin-AP (alkaline phosphatase conjugate) (Roche Applied Science).

Human IgG1 ELISA and HR1 ELISA Assay—The 96-well plates were coated with monoclonal mouse anti-human IgG antibody R10Z8E9 (IgG1 ELISA) or biotinylated T-2324 HR1 peptide (HR1 ELISA) at 0.5 μg/ml in PBS containing 0.5% bovine albumin for 1 h at room temperature. The plates were washed three times with washing buffer (PBS containing 0.05% Tween 20). A standard calibration curve was generated by using serial dilutions of CD4-BFFI in PBS containing 5% cyromolus serum and 0.5% bovine albumin. All serum samples were diluted at 1:20 to 1:20,000 to yield a concentration within the range of quantification: 0.156–10 ng/ml. One hundred μl of diluted standards or samples were added to the plates and incubated for 1 h with gentle shaking. After three washes, 100 μl of 0.2 μg/ml digoxigenin-conjugated mouse monoclonal anti-human IgG antibody R10Z8E9 was added to each well, and the plates were incubated for 1 h with shaking. The plates were washed three times, and 100 μl of horseradish peroxidase-conjugated anti-digoxigenin Fab fragments was added to each well at 50 units/ml final concentration. After a 1-h incubation, the plates were washed three times, and 100 μl of ABTS substrate was added to each well. After a 1-h incubation, the plates were washed three times, and 100 μl of ABTS substrate was added to each well. Color development was monitored every minute at 405 nm.

Cell-Cell Fusion (CCF) Assay—The CCR5-dependent and CXCR4-dependent CCF assays were performed as described before (17). Briefly, Hela-R5 (CCR5-dependent CCF) and Hela-X4 (CXCR4-dependent CCF) cells were plated in 384-well white culture plates at 7,500 cells/well in phenol red-free DMEM supplemented with 10% fetal bovine serum, 1 × penicillin/streptomycin, 300 μg/ml G418, 100 μg/ml hygromycin, and 1 μg/ml doxycycline and incubated at 37 °C overnight to induce the expression of gp160. The target cells CEM-R5 and 5.25 in the phenol red-free growth medium were then added to the plates containing HeLa-R5 and HeLa-X4 cells, respectively, both at 1.5 × 10⁴ cells/well. After a 20-h incubation, 15 μl of Steady-Glo luciferase substrate was added, and the luciferase activity was measured.

Single-cycle Entry Assay and PBMC Antiviral Assay—These assays were performed as described before (9). For the single-cycle assay, pseudotyped viral particles were generated by co-transfecting 293T cells with pNL4-3Δenv (pNL4-3 with a deletion of the envelope gene) and the expression vector pcDNA3.1 (Invitrogen) encoding the envelope gene of NLBal or NL4-3. Cell culture supernatants containing pseudotyped viral particles were harvested and filtered. The equivalent of 1 × 10⁵ relative light units of virus particles was used to infect 25,000 JC53BL cells/well in a total volume of 200 μl. After incubation for 3 days, 50 μl of Steady-Glo luciferase reagent (Promega, Madison, WI) was added and incubated for 5 min, and the plates were read using a Luminoskan (Thermo Electron Corp., Waltham, MA). For the PBMC antiviral assay, pooled human PBMCs from three or more healthy donors were stimulated for 24 h in RPMI 1640 medium supplemented with 2 μg/ml phytohemagglutinin and cultured in RPMI 1640 medium supplemented with 5 units/ml interleukin-2 for at least 48 h prior to the assay. In a 96-well round bottom plate, 1 × 10⁵ PBMCs were infected with 800 pg of p24 of the indicated HIV-1 strain in the presence of serially diluted inhibitor. Plates were incubated for 6 days, and virus production was measured by using p24ELISA (PerkinElmer) according to the manufacturer’s instruction. The IC₅₀ was determined using the sigmoidal dose-response model with one binding site in Microsoft XLfit.

CD4-BFFI Affinity to Cell Surface CD4 Determined by FACS—CD4 mAb 6314 and various CD4-BFFI molecules were covalently labeled with Alexa 488 by using the Alexa Fluor 488 Microscale Protein Labeling kit (Invitrogen) according to the manufacturer’s instructions. In all FACS assays, an isotype control antibody was used to determine the background, which was subtracted from the mean fluorescence intensity (MFI) values for the test antibodies. MAGI cells (1 × 10⁵ cells) were incubated with serially diluted antibodies for 45 min. After washing, the cells were subjected to FACS analysis. MFI values were graphed against antibody concentrations using the one-phase exponential associate curves in GraphPad Prism software (Intuitive Software for Science, San Diego, CA), and the KD values were calculated.

Surface Plasmon Resonance (SPR) Assay—Binding affinities of CD4-BFFI molecules and T-651v peptide to the HIV-1 gp41 HR1 peptide were measured using a BLAcore3000 instrument
CD4-anchoring HIV-1 Entry Inhibitor

(Underlying text)
CD4-anchoring HIV-1 Entry Inhibitor

FIGURE 1. Schematic diagram of expression vector and schematic diagrams and biochemical characterization of CD4-BFFI. A, a map of the eukaryotic expression vector for the expression of CD4-BFFI. The antibody light chain (LC) and COOH-terminally extended heavy chain (HC) are expressed from two identical assembled expression units, including the genomic exon-intron structure of antibody genes. Expression of the light chain and heavy chain is controlled by a shortened intron A-deleted immediate early enhancer and promoter from the human cytomegalovirus and the strong polyadenylation signal from bovine growth hormone. Genetic elements are as follows: immediate early enhancer and promoter from the human cytomegalovirus (CMV-Prom), signal peptide sequence encoding genomic DNA segment (L1–L2), CD4 light chain variable region (VL-CD4), human κ-light chain constant region including a truncated human κ-light chain intron 2 (Cκ), bovine growth hormone polyadenylation signal sequence (BGHpA), CD4 heavy chain variable region (VH-CD4), a genomic human κ-heavy chain constant region containing a truncated/human γ1-heavy chain intron 2 (CH1-Hinge-CH2-CH3), glycine-serine linker composed of three Gly4-Ser repeats (GS-Linker), HIV-1 fusion inhibitor T-651 v peptide (FI), bacterial β-lactamase gene (Apr(t)), and the neomycin resistance structural gene (Neo) flanked by a simian virus 40 promoter (SV40-prom) and polyadenylation signal (SV40-pA). B, CD4-BFFI is composed of the 6314 antibody and two identical T-651v peptides (shown as light blue bars) fused to the COOH-terminal ends of the two heavy chains of 6314 (shown in green) via a (GS)4 linked glycosylation sites (sequons; motif Asn-X-Ser/Thr) are shown as light green circles in the diagram and in blue in the FI sequences. The four mutations in the FIT-651mu of CD4-BFFI are shown in red. C, SDS-PAGE of various CD4-BFFI molecules and 6314. The lanes contain the following samples: molecular weight marker (lane 1); CD4-BFFI (lanes 2 and 7); CD4-BFFI-G2 (lanes 3 and 8); CD4-BFFI-G0 (lanes 4 and 9); 6314 (lanes 5 and 10); and CD4-BFFI (lanes 6 and 11). All samples were treated before loading to the gel. Samples in lanes 2–6 were untreated, and samples in lanes 7–11 were treated with PNGase F (50 milliunits/mg of protein) at 37 °C for 12–24 h.

TABLE 1
Antiviral activity of CD4-BFFI molecules

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 ± S.E. (ng/ml)</th>
<th>IC90 ± S.E. (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>CD4-BFFI</td>
<td>0.5 ± 0.3</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>CD4-BFFI-G2</td>
<td>2.8 ± 0.3</td>
<td>8.6 ± 0.3</td>
</tr>
<tr>
<td>CD4-BFFI-G0</td>
<td>0.1 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>mAb 6314</td>
<td>21.5 ± 2.6</td>
<td>181.9 ± 24.7</td>
</tr>
<tr>
<td>T-651v</td>
<td>Maximum inhibition</td>
<td>55%</td>
</tr>
</tbody>
</table>

potent antiviral activity against both R5 virus NL-Bal and X4 virus NL4-3. CD4-BFFI-G0 proved to be the most potent molecule, with an IC50 value of 2.1 and 10.7 ng/ml against NL-Bal and NL4-3, respectively; CD4-BFFI showed about 2–3-fold lower activity than CD4-BFFI-G0; and CD4-BFFI-G2 was the least potent molecule among the three (Table 1). Interestingly, although mAb 6314 was unable to completely inhibit NL-Bal or NL4-3 infection (maximal inhibition of about 55 and 40%, respectively)
respectively), all three CD4-BFFI molecules showed complete inhibition. Furthermore, all three CD4-BFFIs were more potent than the fusion inhibitor T-651v (Table 1). The difference is even more impressive on a molar basis. The antiviral potency of CD4-BFFI and the FI peptide T-651v against an R5 and an X4 virus were determined in three different antiviral assays: CCF assay, single-cycle HIV entry assay, and PBMC live HIV infection assay. CD4-BFFI was found to be much more potent than T-651v peptide in all three antiviral assays against both R5 and X4 HIV-1 viruses. For example, in the PBMC antiviral assays using live HIV-1 viruses, the IC50 values of CD4-BFFI against NL-Bal and NL4-3 viruses were 0.018 and 0.014 nM, respectively, whereas the IC50 values of T-651v against NL-Bal and NL4-3 viruses were 0.014 and 0.014 nM, respectively.

Of all three bifunctional molecules, CD4-BFFI was found to be the most favorable molecule (optimal balance between antiviral activity and better yield) and was selected for further studies. The potent antiviral activity of CD4-BFFI was confirmed by using two other antiviral assay formats: the CCF assay and the physiologically more relevant PBMC antiviral assays, using replication-competent HIV-1 viruses. In all antiviral assays CD4-BFFI showed complete inhibition and the parental CD4 mAb 6314 showed partial inhibition (Fig. 2). Furthermore, the antiviral potency of CD4-BFFI was found to be superior to the FI peptide T-651v in all three antiviral assays against both R5 and X4 HIV-1 viruses. For example, in the PBMC antiviral assays using live HIV-1 viruses, the IC50 values of CD4-BFFI against NL-Bal and NL4-3 viruses were 0.018 and 0.014 nM, respectively, whereas the IC50 values of T-651v against NL-Bal and NL4-3 viruses were 0.014 and 0.014 nM, respectively.

To understand whether the differences in antiviral potency between the three CD4-BFFI molecules and 6314 are due to changes in their CD4-binding properties, the binding affinity of these molecules was measured. As shown in Fig. 3, 6314 and the three CD4-BFFIs exhibited nearly identical binding affinity to human CD4, with a KD value of 0.17 μM. These data suggest that the observed differences in antiviral activity among three BFFI variants and the parental CD4 mAb 6314 are unlikely to be due to changes in binding affinity to the target receptor CD4.

To further verify that the antiviral potency of CD4-BFFI is due to the FIs linked to the COOH-terminal end of the antibody heavy chains, a CD4-BFFI that carries an inactive T-651v mutant was generated. This T-651mut contains four amino acid mutations (Fig. 1B) at positions that have been shown to be important sites for interaction with HIV-1 gp41 HR1 (2, 3, 7). As shown in Fig. 4A, the chemically synthesized fusion peptide T-651mut was inactive in the single-cycle antiviral assay. The
ability of CD4-BFFImut to interact with HIV-1 gp41 HR1 peptide T-2324 was examined by Western blot analysis. CD4-BFFImut, along with the parental antibody 6314 and CD4-BFFIs, was resolved on a denaturing polyacrylamide gel and probed with a biotinylated HR1 peptide, T-2324, to visualize the antibody heavy chains that bear the functional HR2-derived T-651v peptide. As shown in Fig. 4B, the HR1 probe detected CD4-BFFI, CD4-BFFI-G0, and CD4-BFFI-G2. The probe also detected CD4-BFFImut; however, the signal was dramatically reduced in comparison with CD4-BFFIs that carry the “wild-type” T-651v peptides. Although T-651mut retained residual affinity for HR1 peptide, its affinity may be too low to compete with the native viral HR2 domains that form a highly stable six-helix bundle with HR1 domains during virus-cell fusion (2, 3). This may explain the lack of antiviral activity of T-651mut.

The Western blot analysis also demonstrated that deglycosylation of the CD4-BFFI molecules had no significant effect on binding to HR1 (Fig. 4). In summary, these data suggest that T-651v is able to bind to HR1 peptide when linked to the COOH-terminal end of the CD4 mAb heavy chains, and mutations introduced in T-651mut significantly reduced its ability to interact with the HR1 domain of HIV-1 and consequently the antiviral potency. This was further confirmed by the SPR assay, in which CD4-BFFI showed high binding affinity to T-2324 peptide but CD4-BFFImut displayed only minimal binding affinity (Fig. 5). When CD4-BFFImut was tested in the PBMC antiviral assays, the antiviral potency of CD4-BFFImut was found to be reduced to the level of 6314, against both NL-Bal and NL4-3 viruses. In summary, the FI peptide T-651v in the CD4-BFFI molecule is fully functional, and it is probably the main pharmacophore contributing to the potent antiviral activities of CD4-BFFI.

CD4-BFFI Has High Binding Affinity to HIV-1 gp41 HR1 Peptide—We have shown above by HR1 Western blot analysis that FI T-651v peptide in CD4-BFFI maintained binding ability to the HR1 peptide T-2324. In order to determine the binding affinity of CD4-BFFI to the HR1 peptide, an SPR assay was performed. As shown in Fig. 5, CD4-BFFI demonstrated high binding affinity to the T-2324 HR1 peptides ($K_D = 0.96 \text{ pm}$), resulting from a very fast association and no detectable dissociation within the time frame of the study. Comparing to the T-651v peptide, CD4-BFFI binds 10 times faster and dissociates more than 100-fold more slowly to the HR1 peptide. The off rate observed for CD4-BFFI may be underestimated due to the limitation of the SPR resolution. A
cell lines varied from 203,000 to 14,000 molecules/cell, the CCR5 levels varied from 58,000 to 7,000 molecules/cell, and the CXCR4 levels varied from 4,000 to 8,000 molecules/cell. Despite the marked differences in the expression levels of CD4, CCR5, and CXCR4 on these cells, there was only a marginal difference in potency (2-fold for R5 virus and 6-fold for X4 virus). No correlation between receptor/coreceptor levels and antiviral potency of CD4-BFFI was observed. These results suggest that CD4-BFFI may provide potent protection against HIV-1 viruses irrespective of cell type and the density of receptor and co-receptors.

CD4-BFFI Is Stable in Vivo

—One of the primary goals of creating a second generation HIV-1 fusion inhibitor is to extend the plasma half-life of the F1 peptides. Antibodies are generally very stable proteins and remain in the circulation for weeks. However, fusion of FI peptides to the COOH-terminal end of antibody heavy chains through peptide linkers may significantly affect the pharmacokinetic (PK) properties of the antibody. In addition, the linker and FI peptides may be subjected to proteolysis, resulting in the reduction of antiviral potency. To examine the stability of CD4-BFFI in serum, CD4-BFFI was incubated with monkey serum at 37 °C for various time periods, and the integrity of CD4-BFFI was assessed by HR1 Western blot. As shown in Fig. 6A, even after being exposed to monkey plasma at 37 °C for up to 6 days, the majority of the CD4-BFFI remains intact. Similar stability data were also obtained when incubated with mouse serum (data not shown).

To investigate the in vivo stability of CD4-BFFI, a PK study was conducted in cynomolgus monkeys. Three monkeys were intravenously infused with 10 mg of CD4-BFFI/kg of body weight. Serum levels of CD4-BFFI were monitored by two ELISAs detecting the human IgG1 portion (human IgG1 ELISA) and the T-651v peptide of CD4-BFFI (HR1 ELISA). The HR1 ELISA detects only CD4-BFFI molecules with both human IgG1 and T-651v peptide intact. All three monkeys exhibited very similar nonlinear PK time course. The mean CD4-BFFI serum level, determined by HR1 ELISA, was around 380 μg/ml at 15 min postdosing (Fig. 6B). The levels decreased to about 25 μg/ml by day 7 (168 h), with a calculated terminal t1/2 of 49 h. The CD4-BFFI serum levels determined by a human IgG1 ELISA, which detects both intact and T-651v-truncated forms of CD4-BFFI,

![Figure 5](image_url)

**Figure 5.** Comparative binding affinity of T-651v, CD4-BFFI, and CD4-BFFImut for the HR1 peptide determined by SPR. Shown are BIAcore2000 sensorgrams illustrating binding affinity of the immobilized HR1 peptide at an immobilization level of 200 resonance units to the analytes at five different concentration levels (10 nM (bright green), 5 nM (pink), 2.5 nM (red), 1.25 nM (blue), and 0.625 nM (green)). Binding of the analytes was indicated by a change in response units (RU) over the course of the 60-s injection interval (association phase). After the end of injection, the dissociation was monitored for at least 600 s. A, T-651v peptide. B, CD4-BFFI. C, CD4-BFFImut. D, binding affinity summary table. Binding affinity was determined by analyzing the sensorgram curves obtained with the concentration series, using the BIAevaluation 4.1 software package. The fitting of the data followed the 1:1 Langmuir binding model. The χ² values indicate good applicability of the 1:1 Langmuir model.

### Table 3

<table>
<thead>
<tr>
<th>Cell</th>
<th>Number of receptors/cell</th>
<th>IC₅₀ ± S.E. (μg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>CD4</td>
<td>CCR5</td>
</tr>
<tr>
<td>JC-53BL</td>
<td>202,771</td>
<td>57,862</td>
</tr>
<tr>
<td>MAGI-CCR5</td>
<td>47,121</td>
<td>50,564</td>
</tr>
<tr>
<td>PBMC</td>
<td>13,984</td>
<td>7,406</td>
</tr>
</tbody>
</table>
CD4-anchoring HIV-1 Entry Inhibitor

for serum CD4-BFFI samples taken at 3, 96, and 168 h were determined as 17, 20, and 31 ng/ml, respectively. These results further confirm that CD4-BFFI maintained molecular integrity and antiviral potency \textit{in vivo}.

**DISCUSSION**

The major limitation of current HIV-1 treatment options, including the recently introduced integrase and coreceptor inhibitors, is development of drug resistance in treated patients (4, 23). The treatment strategies involving combinations of two or more agents targeting multiple steps of the HIV-1 life cycle represent the best option to counteract resistance mutations. HIV-1 entry inhibitors are considered an important asset in treating HIV-1 infections (5, 6, 24, 25). Currently, there are two HIV-1 entry inhibitors on the market: the CCR5 antagonist maraviroc (8) and fusion inhibitor ENF (6). The use of maraviroc has been limited due to the requirement of a diagnostic viral tropism test and coverage of only R5 viruses. ENF, a tropism-independent fusion inhibitor, has demonstrated clinical benefit when combined with other antiretrovirals. However, its clinical utility has been limited to the later lines of therapy in patients resistant to other treatments. The key limitation of ENF is its rapid elimination from human plasma, necessitating frequent dosing (90 mg/dose, twice a day) (25, 26). The current investigation was undertaken to explore opportunities to address the limitations of ENF while maintaining its potent activity.

Our previous \textit{in vitro} combination studies revealed that the fusion inhibitor ENF and its structural analogs act highly synergistically with other entry inhibitors, including antibodies against HIV-1 co-receptor CCR5 (27). We have also shown that CCR5 mAb-FI bifunctional inhibitor, consisting of an CCR5 mAb and two fusion peptides, exhibited greater antiviral potency for R5 viruses than either CCR5 mAb, fusion inhibitor alone, or a combination of both. This bifunctional inhibitor, however, failed to prevent the entry of X4 and R5X4 dual-tropic viruses into PBMCs expressing no CCR5 receptors (9). In the current report, we describe a novel bifunctional inhibitor, CD4-BFFI, consisting of an anti-CD4 monoclonal antibody and an HIV-1 fusion inhibitor. CD4-BFFI is not only 100-fold more active than the anti-CD4 antibody and fusion inhibitor, but it also potently inhibited all HIV-1 viruses irrespective of their tropism (Table 2).

were comparable with the levels measured using HR1 ELISA. These data suggest that the CD4-BFFI is stable in monkeys and that the attached linker and fusion peptides remain intact.

In order to understand the saturation status of the CD4 receptors, FACS analyses were performed on blood samples from monkeys following the dosing of CD4-BFFI. PBMC surface CD4 receptors were rapidly bound by CD4-BFFI, with 83% occupancy at 15 min after dosing and 100% occupancy at 30 min. All of the PBMC CD4 receptors remained fully occupied even on day 7, at which point the serum concentration of CD4-BFFI was about 25 μg/ml (Fig. 6D).

To verify that the CD4-BFFI detected by HR1 ELISA is functional, antiviral assays were performed using the serum samples from the monkeys dosed with CD4-BFFI. Serum samples at 3 h, 96 h, and 168 h from each of the three monkeys were serially diluted and tested in the single-cycle HIV-1 entry assay. As shown in Fig. 6C, the dose-response curves of serum samples at the three time points were very similar to that of the CD4-BFFI control. The concentrations of serum CD4-BFFI were calculated based on HR1 ELISA data. The mean IC$_{50}$ for the CD4-BFFI control was 22 ng/ml, and the mean IC$_{50}$ values for serum CD4-BFFI samples taken at 3, 96, and 168 h were determined as 17, 20, and 31 ng/ml, respectively. These results further confirm that CD4-BFFI maintained molecular integrity and antiviral potency \textit{in vivo}.
CD4-anchoring HIV-1 Entry Inhibitor

Mechanistic studies on the CCR5-BFFI molecule suggested that attachment to cell surface CCR5 is required for its antiviral potency, and the FI moieties within CCR5-BFFI were probably the main contributors to the antiviral activity (9). The studies reported here further support this proposed mechanism of action. When the FI peptide was mutated to eliminate its antiviral activity, the antiviral potency of CD4-BFFI carrying the mutated FI peptide (CD4-BFFImm) was reduced to levels similar to the parental CD4 mAb 6314. We also demonstrated the functionality of the FI peptides within CD4-BFFI by measuring binding to the HIV-1 gp41 HR1 peptide in HR1 ELISA and SPR assays. CD4-BFFI was found to bind to the HR1 peptide T-2324 with 100-fold greater affinity than the soluble T-651v peptide. This increased binding is at least partially due to the avidity gained as a result of bivalent binding of the two FI peptides to the T-2324 peptide. The T-651mut peptide alone displayed no antiviral activity; however, when fused to the CD4 mAb 6314, it exhibited detectable binding in both HR1 Western blot and SPR assays. This suggests that the residual affinity of the T-651mut peptide can be strengthened by the avid binding of two peptides fused to one antibody molecule. The enhanced binding affinity to HR1 peptide as a result of increased avidity may be also help explain the much greater antiviral potency of CD4-BFFI than the FI peptide. These results are in line with the published literature demonstrating that three HR2 peptides bind to the HR1 trimer to form the highly stable six-helix bundle (2, 3). In summary, increased local concentration of CD4-BFFI by anchoring to cell surface CD4 receptors and increased binding avidity of the two FI peptides within CD4-BFFI are the most likely mechanisms for the enhanced antiviral potency of CD4-BFFI molecule compared with the FI peptide alone.

In all of the antiviral assays, CD4-BFFI inhibition reached 100% on both R5 and X4 viruses. In contrast, the parental CD4 mAb showed submaximal inhibition for the same two virus isolates (Fig. 2). This was confirmed in a large panel of other R5 and X4 HIV-1 viruses. The reason for the incomplete inhibition is not fully understood. However, it has been reported that HIV-1 isolates from some patients were only partially inhibited by the CD4 mAb TNX-355, and viruses from patients, who developed resistance to TNX-355 treatment, showed markedly reduced maximal inhibition by TNX-355 (28). The fact that 6314 itself cannot fully inhibit all virus strains tested here and elsewhere suggests that the complete inhibition observed with CD4-BFFI is probably a result of the attached T-651v fusion peptide.

In vivo studies in cynomolgus monkeys revealed that the CD4-BFFI molecule is stable in plasma. The CD4-BFFI serum concentration was about 25 μg/ml at day 7 after dosing. This is ~2,000-fold higher than the mean IC_{50} value of CD4-BFFI determined in a PBMC antiviral assay against NL-Bal and NL4-3 viruses (9.9 and 13.6 ng/ml, respectively). The CD4 receptors of the sampled monkey PBMCs were completely occupied with CD4-BFFI for 7 days postadministration (Fig. 6D). Although we cannot accurately predict human CD4-BFFI PK based on monkey PK data, the observed plasma PK behavior of the molecule in monkeys is very encouraging. PK models predict that a human dose of 2 mg/kg of body weight every other week will maintain serum minimal concentration (C_{min}) above mean IC_{50} values.

Published data suggest that synergistic antiviral effects could be achieved when HIV-1 infection inhibitors of different classes (including TNX-355 and ENF) are combined (19, 27). Pill burden has been reported to be one of the reasons of poor patient compliance. Reduction in the number of pills and frequency of dosing may help address this issue. CD4-BFFI, targeting two separate HIV-1 entry steps, high antiviral potency, a potential for better durability of viral suppression, and favorable dosing frequency may address some of the limitations of the current therapies.

In conclusion, we have generated a novel CD4-targeting HIV-1 fusion inhibitor, CD4-BFFI. By anchoring to cell surface CD4 receptors, the CD4 mAb and the two covalently attached HIV-1 FI peptides act synergistically to potently suppress viral entry. CD4-BFFI was able to inhibit the R5 and X4 viruses with equal potency. The use of a humanized CD4 mAb as a scaffold protein for the FI peptide T-651v extended the plasma half-life of the FI peptide from 30 min (ENF) to several days (CD4-BFFI). The increased plasma stability in combination with improved potency may offer greater therapeutic potential to CD4-BFFI as a novel HIV-1 entry inhibitor.

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REFERENCES

17. J. Biomol. Screen 5, 3–15

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