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. 5, 56–65). CD4-BFFI, which consists of two HIV-1 fusion inhibitor (FI) T-651 variant peptides recombinantly fused to the C-terminus of 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 IC_{50} 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 a 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 host cell fusion; thus, it was named bifunctional HIV-1 fusion inhibitor T-651v and T-651v mutant (T-651mut) were chemically synthesized and COOH-terminally amidated fusion inhibitor peptides is inactivated through a point mutation to change Asn to Glu in all BFFI molecules except in BFFI-G2. In CD4-BFFI, the NH2-terminus (NMT) and the other within the peptide. To avoid N-glycosylation that may reduce the antiviral activity of 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 y1-heavy chain gene constant region (containing a truncated human y1-heavy chain intron 2).

For the construction of various CD4-BFFI molecules, the DNA sequences encoding the entire GS peptide linker (GGGGSGGGSSGGGGSS) 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 Nhel 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 H11032 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 peptide 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-QARQLLSGI-LKDQ-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 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, 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 and washed with PBS before the assay. 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 IC50 values were calculated.
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(GE Healthcare). The Sensor Chip SA (streptavidin) was pre-washed by three consecutive 1-min injections of 1 M NaCl in 50 mM NaOH. Next, the biotinylated T-2324 HR1 peptide (Biotin-QARQLGIVQQQNLLRAIEAQHLLQTLTVWGILKQLRILAVELYLDQ-NH2) was immobilized on the SA-coated sensor chip. To avoid mass transfer limitations, the lowest possible value was loaded onto the SA-chip. The HR1 peptide (~200 resonance units) was dissolved in HBS-P buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% (v/v) Surfactant P20). Before the measurements were started, the chip was regenerated with a 1-min pulse of 0.5% (w/v) SDS at a flow rate of 50 μl/min. CD4-BFFI molecules to be analyzed were diluted in HPS-P buffer to various concentrations ranging from 0.625 to 10 nM. The sample contact time was 1 min (association phase). Thereafter, the chip surface was washed with HBS-P for 10 min (dissociation phase). All interactions were performed at exactly 25 °C (standard temperature). During a measurement cycle, the samples were stored at 12 °C. Signals were detected at a detection rate of one signal/s. Samples were injected at increasing concentrations at a flow rate of 50 μl/min over the HR1 coupled biosensor element. The surface was regenerated by 1 min of washing with 0.5% (w/v) SDS solution at a flow rate of 50 μl/min. The equilibrium constants (Kₐ, Kₖₖ), defined as kₐ/kₖₖ, were determined by analyzing the sensorgram curves obtained with several different concentrations, using the BIAevaluation 4.1 software package. Nonspecific binding was corrected by subtracting the response value of the respective CD4-BFFI molecule with the free streptavidin surface from the value of the CD4-BFFI-HR1 interaction. The fitting of the data followed the 1:1 Langmuir binding model.

Determination of Receptor Surface Expression by FACS—Expression levels of CD4, CCR5, and CXCR4 on the surface of MAGI-CCR5, JC53BL, and human PBMCs were determined by FACS (9).

Monkey Pharmacokinetics Study—Three male cynomolgus monkeys weighing 3–5 kg were dosed intravenously with 10 mg of CD4-BFFI/kg of body weight. Blood samples were taken immediately before dosing and 15 min, 30 min, 1 h, 3 h, 8 h, 24 h, 48 h, 72 h, 96 h, and 168 h after dosing. Sera were prepared and stored at −80 °C before use.

PBMC CD4 Occupancy—Whole blood from three monkeys before and after dosing with CD4-BFFI was incubated with mouse anti-human CD4 mAb OKT4-Pacific Blue (BioLegend, San Diego, CA) on ice for 45 min. Red blood cells were removed by incubation in 1 ml of 1x lysis buffer (PharmLyse lysis buffer (10×); BD Biosciences) for 15–20 min followed by centrifugation for 5 min at 300 × g. The pellet was washed with 250 μl of FACS staining buffer (phosphate-buffered saline containing 3% heat-inactivated fetal calf serum, 0.09% (w/v) sodium azide), and CD4 staining on monkey PBMCs was analyzed by FACS. For the calculation of CD4 occupancy, the MFI values from the predose samples were set as 0% CD4 occupancy, and the MFI from the predose samples treated with saturating amount of CD4-BFFI before adding OKT4-Pacific Blue were set as 100% occupancy.

RESULTS

Construction and Characterization of CD4-BFFI—Previous studies with the first generation BFFI containing the HIV-1 FI peptide T-2635 fused to the COOH-terminal end of the heavy chain of a CCR5 mAb demonstrated that the fusion peptide maintains full antiviral activity when conjugated to an antibody (9). The inhibition of viral entry was primarily a result of blocking the virus-cell membrane fusion step. It was demonstrated that anchoring of the T-2635 peptide to the CCR5 co-receptor is essential for maximal antiviral activity, and it was hypothesized that the increased local concentration of the T-2635 peptide at the site of viral entry achieved by antibody-mediated targeting to the CCR5 co-receptor is responsible for the increased antiviral activity. In addition, the CCR5 mAb moiety of BFFI also exerted a direct antiviral effect against R5 viruses. This bifunctional molecule was effective at inhibiting R5 viruses but not R5X4 and X4 viruses in PBMC cultures. The lack of potency against X4 and R5X4 viruses can be explained by the absence of CCR5 receptors on subsets of T lymphocytes (18). Therefore, we speculated that a CD4 mAb-based bifunctional inhibitor targeting all cell types susceptible to HIV-1 infection would prevent entry of HIV-1 irrespective of viral tropism. To test this hypothesis, a bifunctional CD4 mAb-FI molecule was constructed (Fig. 1) based on the variable region of the humanized anti-CD4 antibody TNX-355 (also known as Hu5A8) (10) and the HIV-1 fusion inhibitor peptide T-651v. The HIV entry inhibitor TNX-355 has demonstrated efficacy in cell culture and in patients (11–13). Fusion inhibitor T-651v, instead of T-2635 used in previous studies, was used in the new BFFI molecule. The T-635 is a heavily modified HIV-1 HR2 amino acid sequence designed to stabilize the α-helical conformation, whereas T-651v is derived from a natural HIV-1 HR2 amino acid sequence. The T-651v peptide used in the CD4-BFFI contains an amino acid substitution introduced to eliminate a potential N-linked glycosylation site (Fig. 1), differing itself from the published T-651 sequence. T-651 has shown to provide a high genetic barrier to resistance development (14), and T-651 and T-2635 were equally potent in the first generation CCR5 mAb-based BFFI molecules.4

Initial purification and biochemical characterization of CD4-BFFI molecules lacking N-linked glycosylation sites in T-651v revealed that the molecules tend to form aggregates at high concentrations, resulting in reduced yields of functional protein (data not shown). In an attempt to reduce the aggregation tendency, three variants carrying one or two of the naturally occurring N-glycosylation sites in T-651v were tested. The CD4-BFFI that carries an N-glycosylation site at the NH₂ terminus but not within the T-651v peptide was named CD4-BFFI; the CD4-BFFI that carries both N-glycosylation sites in T-651 was named CD4-BFFI-G2, and the CD4-BFFI that carries no N-glycosylation site in T-651 was named CD4-BFFI-G0. The extent of N-glycosylation was analyzed by comparison of native and deglycosylated (PNGase F-treated) CD4-BFFI samples resolved on SDS-PAGE and/or mass spectrometry (data 4 C. Ji, E. Kopetzki, A. Jekle, K.-G. Stubenrauch, J. Zhang, S. Fischer, N. Cammack, G. Heilek, S. Ries, and S. Sankuratri, unpublished data.)
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FIGURE 1. Schematic diagram of expression vector and schematic diagrams and biochemical characterization of CD4-BFFI. 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 of 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 α-light chain constant region including a truncated human α-light chain intron 2 (C1–α), bovine growth hormone polyadenylation signal sequence (BGHpA), CD4 heavy chain variable region (VH–CD4), a genomic human α1-heavy chain constant region containing a truncated/human γ1-heavy chain intron 2 (CH1-Hinge-CH2-CH3), glycosy-merase linker composed of three GlyGlySer repeats (GS-Linker), HIV-1 fusion inhibitor T-651v peptide (F), bacterial β-lactamase gene (Ap(r)), 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 (GGG)3 linker (GGGGSGGGGGGSG; shown in green). The F1 peptide sequences used in various CD4-BFFI constructs are shown on the right. The potential N-linked glycosylation sites (sequons; motif Asn-X-Ser/Thr) are shown as light green circles in the diagram and in blue in the Fl sequences. The four mutations in the F1-651mut 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-BFFImut (lanes 6 and 11). All samples were reduced 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.

not shown). As shown in Fig. 1C, the heavy chains of CD4-BFFI-G2 containing two N-glycosylation sites migrated more slowly than the CD4-BFFI containing one N-glycosylation site and the CD4-BFFI-G0 containing no N-glycosylation site (Fig. 3C, lanes 2–4). After treatment with PNGase F, the heavy chains of all three molecules were reduced to the same size (Fig. 1C, lanes 7–9). It is also of note that the heavy chains of the parental CD4 mAb 6314 were reduced in size after PNGase F treatment, thus confirming the invariant N-glycosylation of Asp297 in the IgG1 CH2 constant domain. There is no glycosylation site on the light chain of these antibodies, and consequently there is no change in size upon PNGase F treatment. The greater sizes of the deglycosylated CD4-BFFI variant heavy chains compared with the heavy chain of the anti-CD4 antibody 6314 were due to the added GS linker and T-651v sequences (Fig. 1C, compare lanes 7–9 with lane 10).

The three CD4-BFFI molecules were tested in single-cycle antiviral assays. All three CD4-BFFI molecules demonstrated 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), 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. In the physiologically more relevant PBMC antiviral assays, using replication-competent HIV-1 viruses. For example, in the PBMC antiviral assays using live HIV-1 viruses, the IC_{50} values of CD4-BFFI against NL-Bal and NL4-3 viruses were 0.018 and 0.014 nM, respectively, whereas the IC_{50} values of T-651v against NL-Bal and NL4-3 viruses were 1.32 and 0.78 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 IC_{50} values of CD4-BFFI against NL-Bal and NL4-3 viruses were 0.018 and 0.014 nM, respectively, whereas the IC_{50} values of T-651v against NL-Bal and NL4-3 viruses were 1.32 and 0.78 nM, respectively (Table 2).

Antiviral Potency of CD4-BFFI Is Dependent on Its FI Entity—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 K_{D} value of 0.17 μg/ml. 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
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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 peptide (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 ($K_D = 0.96 \mu M$), 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.

Antiviral Potency of CD4-BFFI Is Independent of Cell Surface Receptor and Coreceptor Levels—Our previously published mechanism of action studies with CCR5-BFFI suggested that the enhanced antiviral potency of BFFI compared with T-2635 was mainly due to anchorage of BFFI to the coreceptor CCR5, thus allowing the antibody-tethered FI peptide to block HIV-1 fusion efficiently (9). Data from the current study and other data\(^5\) suggest that CD4-BFFI inhibits HIV-1 entry by a similar mechanism. CD4 and the coreceptors CCR5 and CXCR4 are expressed at different levels in the various lymphocyte subsets. Published data suggest that levels of HIV-1 receptor and coreceptor on target cells may greatly affect HIV-1 entry kinetics and the activity of entry inhibitors (20–22). Therefore, it is important to determine the influence of receptor and coreceptor levels on the antiviral potency of CD4-BFFI against R5 and X4 viruses.

The expression levels of CD4, CCR5, and CXCR4 on J53BL, MAGI-CCR5, and human PBMCs were determined by FACS analysis. As shown in Table 3, the CD4 levels among these three

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\(^5\) A. Jekle, E. Chow, E. Kopetzki, C. Ji, M. Yan, R. Nguyen, S. Sankuratri, N. Camack, and G. Heilek, submitted for publication.
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 FI 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 ng/ml at 15 min postdosing (Fig. 6B). The levels decreased to about 25 ng/ml by day 7 (168 h), with a calculated terminal $t_{1/2}$ 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,
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 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 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).
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-BFFImut) 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 avidity 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 thus may 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 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 IC50 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 (Cmin) above mean IC50 values.

Published data suggest that synergistic antiviral effects could be achieved when two HIV-1 entry 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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