Antiviral Research 83 (2009) 257–266

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

CD4-BFFI: A novel, bifunctional HIV-1 entry inhibitor with high and broad antiviral potency

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ARTICLE INFO

Article history:
Received 22 December 2008
Received in revised form 29 March 2009
Accepted 16 June 2009

Keywords:
HIV-1
Fusion inhibitor
CD4 receptor
Antibody

ABSTRACT

Resistance to antiretroviral drugs is a common problem in the treatment of HIV-1-infected patients. To overcome resistance, we generated a novel, bifunctional HIV-1 entry inhibitor by combining the anti-CD4 monoclonal antibody (mAb) 6314 with a fusion inhibitor similar to T-651 (anti-CD4 mAb based BiFunctional Fusion Inhibitor, CD4-BFFI). CD4-BFFI has potent antiviral activity against a multitude of HIV-1 isolates independent of their co-receptor usage and genetic background. It has higher antiviral potency compared to the fusion inhibitor T-651 or the anti-CD4 mAb 6314 used independently. More importantly, every HIV-1 strain tested was fully inhibited by CD4-BFFI while many strains were only partially inhibited by 6314. CD4-BFFI also retained antiviral potency against virus strains resistant to two fusion inhibitors, a CCR5 antagonist and an anti-CCR5 mAb. Pre-incubation of cells with a saturating concentration of anti-CD4 mAbs reduced the antiviral potency of CD4-BFFI, suggesting that binding of CD4-BFFI to the cell surface via its CD4 mAb portion is required for the antiviral potency of its fusion inhibitor moiety. Collectively, we present a novel HIV-1 inhibitor with a dual mode of action and excellent antiviral potency against wildtype and entry-inhibitor resistant virus strains suggesting that CD4-BFFI may have a high barrier to resistance.

1. Introduction

The main cellular targets of the human immunodeficiency virus (HIV-1), the etiological agent of AIDS, are CD4-lymphocytes and macrophages. HIV-1 entry requires the interaction of the viral gp120 protein with the CD4 receptor and one of two major co-receptors, CCR5 or CXCR4 (Berger et al., 1998, 1999). CCR5-using virus strains (R5-tropic) predominate during primary infection and the early asymptomatic phase whereas CXCR4-using (X4-tropic) viruses appear during later disease stages in about 50% of HIV-1-infected individuals (Berger et al., 1999; Connor et al., 1997). Emergence of X4-tropic virus is associated with a steeper decline in CD4 T-cells and an accelerated disease progression (Berger et al., 1999; Connor et al., 1997; Schuitemaker et al., 1992). Dual-tropic viruses, which can use both co-receptors, are evolutionary and functional intermediates between R5- and X4-tropic viruses (Berger et al., 1999; Connor et al., 1997; Schuitemaker et al., 1992; Tasca et al., 2008).

HIV-1 entry is a multi-step process (reviewed in Chan and Kim, 1998; Gallo et al., 2003; Moore and Doms, 2003); Initial contact is made between the viral gp120 surface protein trimer and the CD4 receptor, causing conformational changes in gp120, and exposing conserved regions important for co-receptor binding. Subsequent binding to the co-receptor results in a second conformational change allowing insertion of the gp41 envelope subunit fusion peptide into the host cell membrane. As gp120 dissociates from gp41, the heptad repeat (HR) 2 in each of the three gp41 units condenses with their respective HR1 domains, forming a 6-helix bundle and bringing viral and host membrane into close proximity. Finally, a fusion pore is formed, allowing entry of the HIV-1 capsid into the target cell.

This multi-step process offers several targets of therapeutic intervention (reviewed in Este and Telenti, 2007; Moore and Doms, 2003). CD4 engagement can be inhibited by either small molecules binding to the CD4 binding domain on the viral gp120 protein such as BMS-806 (Madani et al., 2004; Wang et al., 2003) or the CD4-IgG chimera Pro542 (Allaway et al., 1995) or protein inhibitors specific to the CD4 receptor such as the anti-CD4 monoclonal antibody Ibalizumab (formerly known as TNX-355) (Burkly et al., 1992; Kuritzkes et al., 2004; Jacobson et al., 2008). Small molecule antagonists against both co-receptors CCR5 and CXCR4 are in development; the CCR5 inhibitor maraviroc (Selzentry) was approved by the Food

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doi:10.1016/j.antiviral.2009.06.005

and Drug Administration in 2007 (Fatkenheuer et al., 2005; Wood and Armour, 2005), and others (vicriviroc, INCB9471) are in clinical trials (Erickson-Viitanen et al., 2008; Ogert et al., 2008; Tagat et al., 2004). Similarly, small molecule antagonists against CXCR4 have been shown to have antiviral activity in vitro and in vivo (De Vreese et al., 1996; Hendrix et al., 2004; De Clercq et al., 1994). In addition to the small molecule co-receptor antagonists, several anti-CCR5 mAbs are in development (Ji et al., 2007; Lalezari et al., 2006; Trkola et al., 2001). Finally, the fusion inhibitor enfuvirtide (T-20, Fuzeon), which blocks the formation of the 6-helix bundle, was the first entry inhibitor approved in 2003 (Chen et al., 1995; Kilby et al., 1998).

One of the challenges that entry inhibitors, independent of their mechanism of action, share with other antiretroviral drugs, is rapid resistance development: two amino acid changes in the V3 loop of gp120 can confer resistance against the CCR5 antagonists maraviroc, vicriviroc or INCB9471 (Erickson-Viitanen et al., 2008; Marozsan et al., 2005; Pugach et al., 2007; Westby et al., 2007). Likewise, resistance against the fusion inhibitor T-20 arises quickly in both cell culture experiments and in the clinic (Greenberg and Cammack, 2004; Rimsky et al., 1998; Wei et al., 2002). Viruses isolated from patients treated for nine weeks with the anti-CD4 mAb Ibalizumab in a phase 1b trial had reduced susceptibility to Ibalizumab (Jacobson et al., 2004, 2008). To circumvent the problem of a low genetic hurdle to resistance, we previously engineered a novel anti-HIV-1 compound that combined the antiviral activity of two entry inhibitors: this bifunctional fusion inhibitor (BFFI) consisted of an anti-CCR5 mAb covalently linked to the fusion inhibitor T-2635 (Kopetzki et al., 2008). We hypothesized that combining two entry inhibitors with different mechanisms of action would increase the antiviral potency and durability. While the antiviral potency of BFFI against R5-tropic virus strains was increased compared to the CCR5 mAb or T-2635 alone, it was inactive against X4-tropic or dual-tropic viruses. We used an anchoring model to reason that the antiviral activity against X4-tropic virus depends on BFFI's ability to bind via its anti-CCR5 mAb portion to the target cell. Cells that do not express CCR5 can therefore not be protected from X4-tropic HIV-1 infection using this particular BFFI molecule (Kopetzki et al., 2008).

Based on this concept, we designed a new bifunctional HIV-1 entry inhibitor, which has high antiviral activity against R5-, X4- and dual-tropic virus strains. This novel molecule, called CD4-BFFI, consists of an anti-CD4 mAb covalently linked to two molecules of a modified version of the fusion inhibitor T-651(Ji et al., 2009). The exchange of the antibody portion of the BFFI molecule from anti-CCR5 to anti-CD4 relieved the co-receptor restriction of the original BFFI. In addition, this second generation BFFI is active against viruses resistant to fusion inhibitors, CD4 antibodies and CCR5 antagonists and antibodies.

2. Materials and methods

2.1. Cell lines, virus strains and other reagents

JC53BL (TZM-bl) cells were obtained from the NIH AIDS Research and Reference Reagent program. 293T human embryonic kidney cells were obtained from ATCC, Manassas, VA. Human PBMC were obtained from AllCells (Emeryville, CA), stimulated for one day in PBMC media (RPMI-1640 media containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium-pyruvate, 0.1 mM MEM non-essential amino acids) supplemented with 2 μg/ml phytohemagglutinin (all Invitrogen) and maintained in PBMC media containing 5 units/ml human IL-2 (Roche Applied Science, Indianapolis, IN).

HIV-1 strains NL4-3, RU570, 301567, 92HT599 and 92US715 were obtained from the NIH AIDS Research and Reference Reagent program. YU2.c was obtained from Dr. G. Shaw, University of Birmingham, Alabama. JRCSF was obtained from Dr I. Chen, University of California, Los Angeles. NLBal was obtained from Roche Welwyn, UK. Virus strains Bal and 89.6 were purchased from ABI, Colombia, MD. CC1/85 was a gift from Dr. C. Stoddart (The J. David Gladstone Institutes, San Francisco, CA). W969-2, W969-5 and W969-7 were a gift from Dr. D. Richman, University of California, San Diego. The fusion-inhibitor resistant viruses described in Table 4 were a gift from Dr. M. Greenberg (Trimeris, Morrisville, NC). The viruses used in Table 2 as well as BK132 and BZ162 were obtained from BBI Diagnostics, Milford, MA.

Envelope clones 93TH6.9, JRFL, SF162 and HXB2 were obtained from the NIH AIDS Research and Reference Reagent program. Envelope clones 2148#58, 1013#3, CS81-69-E09, CS81-4-G0 and C597-8-H0 were cloned from plasma from HIV-1-infected plasma obtained from Biocollections Worldwide, Miami, FL.

The N-terminally acetylated and C-terminally amidated T-651 and T-1144 peptides were a gift from Dr. M. Greenberg (Trimeris, Morrisville, NC), synthesized as described in Davison et al. (2006) and Dwyer et al. (2007). The N-terminally acetylated and C-terminally amidated modified version of T-651 (amino acid sequence: Ac-**N**MTWMEWDREINQYTSLIHSLIEESQNQQEKNEQELL) was custom-synthesized at Biopeptide co., Inc., San Diego, CA). The anti-CD4 antibodies RPA-T4 and MEM115 and the mouse isotype control antibody were purchased from Abcam, Cambridge, MA. AMD3100 was synthesized at Roche Palo Alto as described previously (De Clercq et al., 1992).

2.2. Expression plasmids

The amino acid sequences used for the variable domains (VL and VH) of the anti-CD4 mAb 6314 and CD4-BFFI have been described (Reimann et al., 1997). Antibody light and heavy chain genes are expressed from 2 identical assembled expression units including the genomic exon–intron structure of antibody genes. 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. The structural gene of the CD4-BFFI light chain is assembled by fusing a chemically synthesized CD4 antibody variable light chain cDNA at the 5′-end with a DNA segment encoding a murine immunoglobulin heavy chain signal sequence including a signal sequence intron (L1, intron, L2) and at the 3′-end with a DNA segment containing a splice donor site and a unique BsmHI restriction site. The BsmHI restriction site enables the fusion to the human κ-light chain gene constant region including a truncated human κ-light chain intron 2. A unique BsmHI restriction site, which was engineered into the L2 signal sequence, was used for joining of the CD4 mAb encoding variable light chain cDNA. The structural gene of the CD4-BFFI heavy chain is assembled by fusing a chemically synthesized CD4 antibody variable heavy chain cDNA at the 5′-end with a DNA segment encoding a murine immunoglobulin heavy chain signal sequence including a signal sequence intron (L1, intron, L2) and at the 3′-end with a DNA segment containing a splice donor site and a unique XhoI restriction site. The XhoI restriction site enables the joining to the genomic human γ1-heavy gene constant region including a truncated murine/human γ1-heavy chain hybrid intron 2. A unique BsmHI restriction site, which was engineered into the L2 signal sequence, was used for joining the CD4 mAb encoding variable heavy chain cDNA. To enable the C-terminal fusion of the peptide linker and the peptide fusion inhibitor to the C-terminus of the heavy chain (and also the subsequent substitution) a unique HindIII and NheI restriction site is inserted (i) into the DNA-sequence encoding the heavy

chain CH3 domain while maintaining the encoding amino acid sequence and (ii) the DNA sequence joining the open reading frame of the heavy chain fusion protein and the bGH polyadenylation signal, respectively. The CD4 antibody cassette is followed by a Gly–Ser linker peptide composed of three Gly_4Ser repeats and a modified version of the HIV-1 inhibitory peptide T-651 which is derived from the gp41 ectodomain of HV1B8 HIV-1 reference strain (BH8 isolate; position: 610-656; residue numbering is based on the envelope polyprotein gp160 precursor (Ratner et al., 1985). The potential N-glycosylation site (AsnTyrThr) within the peptide is removed through a point mutation (GlnThyThr). The amino acid sequence of the modified T-651 fusion inhibitor entity is: **N**M**T**WMEWDREIN**Q**Y**T**SLIHSLIEESQNQQEKNEQELL). The expression plasmid for the CD4 mAb is identical to the one for CD4-BFFI without the Gly–Ser linker and the fusion inhibitor peptide.

2.3. Expression, purification and analytical characterization of proteins

CD4-BFFI and the anti-CD4 mAb 6314 were expressed, purified and characterized as described (Kopetzki et al., 2008).

2.4. Single cycle entry assay

To generate pseudotyped HIV-1 particles, 293T cells were co-transfected with pNL4–3Δenv (pNL4-3 with a deletion of the envelope gene) and an expression vector encoding the indicated envelope gene. Cell culture supernatants containing pseudotyped viral particles were harvested, filtered, stored at −80 °C and titered on JC53BL cells. To test for antiviral potency, compounds were diluted in quadruplicates in white 96-well-plates (Greiner Bio-one, Frickenhausen, Germany). The equivalent of 1×10^5 relative light units of virus particles were used to infect 25,000 JC53BL cells/well in a total volume of 200 μl. After incubation for 3 days, 50 μl Steady-Glo® luciferase reagent (Promega, Madison, WI) was added and the plates were read using a Luminoskan (Thermo Electron Corporation, Waltham, MA) after 5 min. The IC_{50} was determined using the sigmoidal dose–response model with one binding site in Microsoft XLfit.

2.5. Antiviral assay

Antiviral potencies using replication-competent virus strains were generated by infecting 25,000 JC53BL cells as described for the single cycle entry assay. Viruses were amplified in PBMC and viral replication was monitored by infecting JC53BL cells and analyzing subsequent luciferase read-out. Cell culture supernatants containing viral particles were harvested, filtered and stored at −80 °C. To test for antiviral potency, the protocol for the single cycle entry assay was followed.

2.6. Synchronized infection experiments

For the time course infection study, JC53BL cells (2×10^4/well) were seeded in 96-well plates overnight and infected with HIV-1 RF virus (equivalent to 10^5 RLU/well) by centrifugation at 2400 rpm for 2 h at 4 °C. Cells were washed twice with cold media. The temperature arrest was released by addition of 150 μl warm (37 °C) media to each well. At the indicated time points, 50 μl of pre-warmed entry inhibitors diluted in media were added to a final concentration of 5 μg/ml. Luciferase activity was measured 72 h post-infection as described above.

2.7. PBMC assay

Human PBMC mixed from three or more healthy donors were stimulated for 24 h in media supplemented with 2 μg/ml phytohemagglutinin and cultured in media supplemented with 5 Units/ml IL-2 for at least 48 h prior to the assay. In a 96 well round bottom plate, 1×10^5 PBMC were infected with 800 pg of p24 from the indicated HIV-1 strain in the presence of serially diluted inhibitor. Plates were incubated for 6 days at 37 °C. Virus production was measured using p24 ELISA (PerkinElmer) according to the manufacturer's instruction. IC_{50} was determined using the sigmoidal dose–response model with one binding site in Microsoft XLfit.

2.8. Cytotoxicity assay

Human PBMC were prepared as described under 2.7. In a 96 well round bottom plate, 1×10^5 PBMC were incubated with serially diluted inhibitor for 7 days at 37 °C. Cell viability was measured by adding 50 μl CellTiter-glo® (Promega). Plates were analyzed using a Luminoskan (Thermo Electron Corporation, Waltham, MA) after 5 min. The CC_{50} was determined using the sigmoidal dose–response model with one binding site in Microsoft XLfit.

3. Results

3.1. Broad antiviral activity of CD4-BFFI

Previously, we described a bifunctional HIV-1 entry inhibitor (BFFI) consisting of an anti-CCR5 mAb covalently linked to two molecules of the fusion inhibitor T-2635 (Kopetzki et al., 2008). While in peripheral blood mononuclear cell (PBMC) assays this molecule had increased antiviral activity against R5 tropic virus strains, it was inactive against X4 and dual tropic viruses since it requires CCR5 expression on the target cells for binding and subsequent inhibition of HIV-1 entry through its fusion inhibitor moiety. A large percentage of PBMC express CD4 and CXCR4, but not CCR5 (Bleul et al., 1997; Schweighardt et al., 2004) and these cells are therefore not protected by BFFI from X4-infection (Kopetzki et al., 2008). We designed a new bifunctional HIV-1 entry inhibitor that couples two fusion inhibitor molecules similar to T-651 to the C-terminal ends of an anti-CD4 mAb (CD4-BFFI, Fig. 1; Ji et al., 2009).

Based on the anchoring model for the original BFFI, we expected that the new CD4-BFFI molecule would have antiviral activity against R5, X4 and dual-tropic virus strains since its anti-CD4 mAb portion should guarantee binding to all target cells of HIV-1, independent of co-receptor usage. We determined the antiviral activity of CD4-BFFI in comparison to the fusion inhibitor T-651 or the anti-CD4 mAb 6314 in a single cycle entry assay using virions pseudotyped with envelopes from five R5-tropic, four X4-tropic and three dual-tropic virus strains (Table 1). The two amino acids differences between T-651 and the modified version of T-651 used in CD4-BFFI did not affect its antiviral potency (data not shown). CD4-BFFI was more active than T-651 or CD4 mAb 6314 against

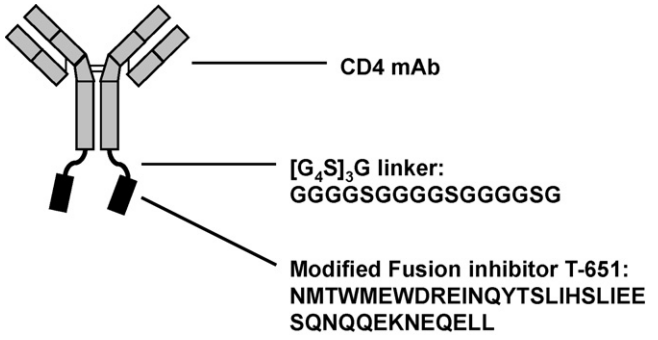

Fig. 1. Schematic diagram of CD4-BFFI: CD4-BFFI is composed of two copies of a fusion inhibitor similar to T-651, which are covalently connected to the CD4 mAb 6314 via $[G_4S]_3G$ linkers.

Table 1
Antiviral potency of the fusion inhibitor T-651, the CD4 mAb 6314 and CD4-BFFI against HIV particles pseudotyped with different envelopes in a single cycle entry assay.

Virus	Tropism	IC_{50} [ng/ml]		
		T-651	CD4 mAb 6314	CD4-BFFI
NLBal	R5	4.4 ± 2.9	547.9 ± 199.4	3.1 ± 2.2
W969-5#1	R5	69.1 ± 25.7	73% max. inhib.[a]	7.7 ± 2.3
93TH6.9	R5	345.2 ± 253.0	80% max. inhib.[a]	10.2 ± 2.7
JRFL	R5	88.9 ± 11.8	41% max. inhib.[a]	5.8 ± 1.2
SF162	R5	306.8 ± 25.1	43% max. inhib.[a]	10.5 ± 0.8
NL4-3	X4	29.7 ± 0.33	51% max. inhib.[a]	42.1 ± 4.5
HXB2	X4	10.9 ± 2.2	49% max. inhib.[a]	7.5 ± 2.5
2148#58	X4	7.3 ± 2.49	62% max. inhib.[a]	5.7 ± 1.1
1013#3	X4	7.5 ± 2.34	33% max. inhib.[a]	5.7 ± 2.2
CS81-69-E09	R5X4	25.2 ± 6.8	34% max. inhib.[a]	6.2 ± 0.8
CS81-4-G0	R5X4	25.8 ± 6.5	69% max. inhib.[a]	6.3 ± 0.6
C597-8-H0	R5X4	22.9 ± 5.3	44.6 ± 13.1	10.0 ± 2.1
Geo Mean[b] [ng/ml]	all	30.9 ± 118.6	n.a.	7.9 ± 10.3
Geo Mean[b] [nM]	all	6.9 ± 26.5	n.a.	0.04 ± 0.07

Shown are the average IC_{50} ± SD from 3 or more independent experiments.
[a] The maximal inhibition is shown instead of the IC_{50} in cases were the dose–response curves show a plateau at levels less than 90%.
[b] Geometric mean ± SD for all virus strains.

all twelve viruses independent of their co-receptor usage. It inhibited the twelve virus strains tested with a geometric mean IC_{50} of 0.04 ± 0.07 nM compared to 6.9 ± 26.5 nM for the fusion inhibitor T-651 (Table 1). In contrast, only two virus strains were fully inhibited by the anti-CD4 mAb 6314, while all others had a maximum inhibition of 33–80% (Table 1). Incomplete dose–response curves have been described before for virus strains resistant to entry inhibitors such as CCR5 antagonists and anti-CD4 antibody, consistent with an allosteric mechanism of inhibition (Jacobson et al., 2004, 2008; Pugach et al., 2007; Westby et al., 2007). Pharmacologically more important, CD4-BFFI is also more active than T-651 on a weight basis: It inhibited the viruses tested with a geometric mean IC_{50} of 7.9 ± 10.3 ng/ml compared to 30.9 ± 118.6 ng/ml for T-651 (Table 1).

We expanded the testing of the antiviral potency of CD4-BFFI to 20 replication-competent HIV-1 strains from all major clades (four isolates from clade C, three isolates from clade B, two isolates each from clade A, D, F, group O and the common recombinant forms CRF01_AE and CRF02_AG and one isolate from clade H). Similar to the results presented in Table 1, CD4-BFFI inhibited all 20 virus strains with a low IC_{50} of 26.8 ± 10.8 ng/ml (geometric mean of all 20 strains ± standard deviation, range 12.7–55.1 ng/ml) (Table 2). The IC_{50} for T-651 was about 3-fold higher (78.2 ± 79.0 ng/ml, range 26.3–310.3 ng/ml). The anti-CD4 mAb 6314 fully inhibited only nine of the twenty virus strains, while the eleven other strains were only partially inhibited. The geometric mean IC_{50} for the subset of nine virus strains that were fully inhibited by the CD4 mAb 6314 is 90.48 ± 14.95 ng/ml. On a molar basis, CD4-BFFI is about 100-fold more active than T-651: It inhibited all 20 viruses with an IC_{50} of 0.17 ± 0.07 nM compared to 17.45 ± 17.63 nM for T-651. 6314 inhibited nine virus strains with an IC_{50} of 0.61 ± 0.10 nM, but did not fully inhibit the remaining eleven virus strains.

Table 2
Antiviral potency of the fusion inhibitor T-651, the CD4 mAb 6314 and CD4-BFFI against replication-competent HIV-1 strains from different clades using the antiviral assay.

Virus	Clade/group	IC_{50} [ng/ml]		
		T-651	CD4 mAb 6314	CD4-BFFI
UG273	A	106.6 ± 31.0	32% max. inhib.[a]	55.1 ± 18.4
UG275	A	39.0 ± 1.5	71.3 ± a2.3	25.5 ± 6.2
CM235	CRF01_AE	240.1 ± 24.4	45% max. inhib.[a]	43.3 ± 21.8
ID17	CRF01_AE	205.0 ± 22.7	71.5 ± 35.6	37.0 ± 16.5
DJ258	CRF01_AG	62.8 ± 8.4	41% max. inhib.[a]	19.5 ± 12.5
DJ263	CRF01_AG	310.3 ± 63.9	144.a ± 40.9	43.9 ± 1.2
BK132	B	43.3 ± 6.9	106.4 ± 9.8	22.5 ± 0.7
BZ167	B	71.6 ± 6.2	182.5 ± 6.5	44.2 ± 3.2
CM237	B	41.5 ± 5.1	49% max. inhib.[a]	20.7 ± 4.7
ETH2220	C	191.6 ± 17.7	158.0 ± 91.1	23.9 ± 4.0
SM145	C	164.0 ± 3.6	48.0 ± 0.4	29.1 ± 0.2
DJ259	C	48.9 ± 19.7	38% max. inhib.[a]	19.5 ± 2.4
UG268	C	49.5 ± 11.1	29% max. inhib.[a]	28.5 ± 2.7
UG274	D	80.2 ± 29.5	22% max. inhib.[a]	38.6 ± 16.8
UG270	D	35.7 ± 3.3	45% max. inhib.[a]	27.5 ± 0.5
BZ162	F	36.1 ± 14.3	31% max. inhib.[a]	25.8 ± 0.5
BCI-R07	F	47.0 ± 1.0	29% max. inhib.[a]	19.3 ± 9.1
BCF-KITA	H	50.3 ± 16.3	32% max. inhib.[a]	28.1 ± 1.5
BCF-06	O	26.3 ± 17.2	52.2 ± 2.2	12.7 ± 4.5
BCF-11	O	151.5 ± 107.1	61.0 ± 32.7	17.4 ± 11.0
Geo Mean[b] [ng/ml]	All	78.2 ± 79.0	90.48 ± 14.95 [c]	26.8 ± 10.8
Geo Mean[b] [nM]	All	17.45 ± 17.63	0.61 ± 0.10 [c]	0.17 ± 0.07

Shown are the average IC_{50} ± SD from three or more independent experiments.
[a] The maximal inhibition is shown instead of the IC_{50} in cases were the dose–response curves plateau at levels less than 90%.
[b] Geometric mean ± SD.
[c] For the anti-CD4 mAb 6314, the geometric mean was calculated only for the nine viruses that were fully inhibited (UG275, ID17, DJ263, BK132, BZ167, ETH2220, SM145, BCF-06 and BCF-11).

Table 3
Antiviral potency of the fusion inhibitor T-651, the CD4 mAb 6314 and CD4-BFFI against different HIV-1 isolates in the PBMC assay.

Virus	Tropism	IC_{50} [ng/ml]			
		T-651	CD4 mAb 6314	CD4-BFFI	*n*
JRCSF	R5	211.4 ± 49.7	35.1 ± 22.9	7.6 ± 0.8	3
NLBal	R5	26.7 ± 17.2	61% max. inhib.[a]	2.7 ± 1.8	6
RU570	R5	22.3 ± 10.9	27.8 ± 1.0	4.1 ± 2.3	3
Bal	R5	217.8 ± 69.4	73% max. inhib.[a]	15.1 ± 9.4	3
CC1/85	R5	159.8 ± 86.5	20% max. inhib.[a]	16.7 ± 9.4	3
301567	R5	47.1 ± 21.6	18.0 ± 14.2	11.5 ± 9.0	3
89.6	R5X4	260.8 ± 88.4	26.0 ± 13.9	11.8 ± 5.5	3
NL4-3	X4	62.0 ± 55.1	80% max. inhib.[a]	1.8 ± 0.9	5
92HT599	X4	282.9 ± 104.8	59% max. inhib.[a]	13.7 ± 1.7	3
BK132	X4	344.8 ± 19.1	60% max. inhib.[a]	30.9 ± 0.9	3
BZ162	X4	216.6 ± 46.1	74% max. inhib.[a]	15.8 ± 9.9	3
Geo mean [ng/ml][b]	All	119.4 ± 112.7	n.a.	9.1 ± 8.2	n.a.
Geo mean [nM][b]	All	26.6 ± 25.1	n.a.	0.06 ± 0.05	n.a.

Shown are the average IC_{50} ± SD from 3 or more independent experiments.
[a] The maximal inhibition is shown instead of the IC_{50} in cases were the dose–response curves plateau at levels less than 90%.
[b] Geometric mean ± SD for all virus strains tested.

We next verified the antiviral activity of CD4-BFFI against R5, X4 and dual-tropic viruses in a PBMC assay. PBMC are the natural host cells for HIV-1 and express biologically relevant levels of CD4, CCR5 and CXCR4 (Bleul et al., 1997). In the PBMC assay, CD4-BFFI blocked infection with all eleven HIV-1 strains tested, independent of their co-receptor usage with an IC_{50} of 9.1 ± 8.2 ng/ml (geometric mean ± SD), compared to 119.4 ± 112.7 ng/ml for T-651 (Table 3). Similar to our observations in the single cycle entry assay, only four of the eleven viruses were fully inhibited by the anti-CD4 mAb 6314, while the remaining seven strains were only partially inhibited (Table 3). Due to its high molecular weight, the superior antiviral potency is even more pronounced on a molar basis: the geometric mean IC_{50} of 0.06 ± 0.05 nM for CD4-BFFI is about 440-fold lower than the one for T-651 (26.6 ± 25.1 nM). In addition, neither CD4-BFFI nor the anti-CD4 mAb 6314 or T-651 is cytotoxic in PBMC at concentrations up to 50 μg/ml while AZT showed dose-related cytotoxicity with a CC_{50} of 91.3 ± 4.74 μM (Fig. 2).

3.2. Increased antiviral potency of CD4-BFFI compared to T-651 and 6314

Combining the results of Tables 1–3, the CD4-BFFI molecule is highly active against a total of 41 virus strains independent of their tropism and geographic origin and independent of the antiviral assay system. In addition, CD4-BFFI is also more active than its components individually, T-651 and 6314. We carried out both single cycle entry (Fig. 3A) and PBMC assays (Fig. 3B–D) to investigate if the high antiviral activity of CD4-BFFI could also be achieved by simply mixing the anti-CD4 mAb 6314 with T-651 at a molar ratio of 1:2. In both assays, CD4-BFFI exceeds the antiviral activity of the T-651/6314 mixture (Fig. 3). To ensure that the strong synergistic effect is not due to the weak inhibition of 6314 against a particular virus strain, we used three different virus strains with different sensitivity to 6314; no significant antiviral potency against CC1/85, partial inhibition against NLBal and complete inhibition against 301567. However, independent of the virus, the antiviral activity of CD4-BFFI was superior to the T-651/6314 mixture (Fig. 3B–D).

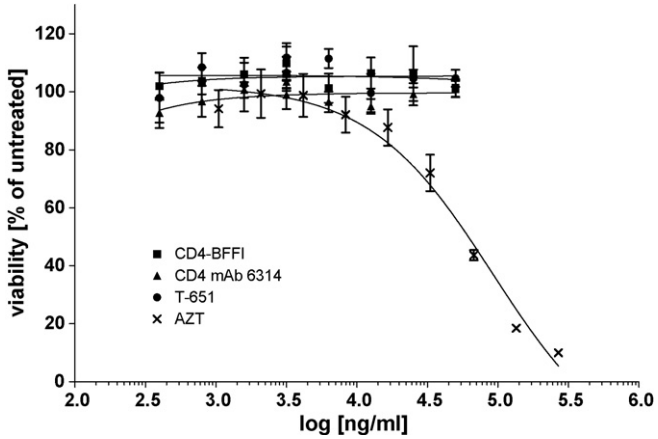

Fig. 2. CD4-BFFI, CD4 mAb 6314 and T-651 and are not cytotoxic to PBMC at concentrations up to 50 μg/ml. PBMC were incubated for seven days with increasing concentrations of CD4-BFFI (■), the anti-CD4 mAb 6314 (▲), T-651 (●) and AZT (×). The average and standard deviation of two independent experiments carried out in triplicates are shown.

3.3. Inhibition of entry-inhibitor-resistant HIV-1 variants by CD4-BFFI

Besides increased antiviral potency, we also expected that combining two entry inhibitors into one molecule would provide an entity with antiviral potency against virus strains resistant to entry inhibitors with single mode of action. We tested the antiviral activity of CD4-BFFI against four virus strains that were selected during *in vitro* passaging experiments against the fusion inhibitor T-20 (enfuvirtide, Fuzeon) and one strain resistant against T-1144 (Davison et al., 2006). As expected, the T-20 resistant strains NL178, DH12RF178, Re4 and RFRD178 were not inhibited by T-20, while their respective parental wildtype strains 3′GIV, DH012, IIIB and RF remained sensitive to T-20 (Table 4). Similarly, T-1144 had a 9-fold reduced activity against 098-1144 compared to the corresponding wildtype strain 098 (Table 4). In contrast, CD4-BFFI blocked all five fusion-inhibitor resistant virus strains without any significant loss in activity compared to the wildtype strains (Table 4). Interestingly, while 098-1144 had a 12-fold loss in susceptibility to T-651, CD4-BFFI had only a minor 2-fold loss (Table 4), suggesting that the 6314 antibody portion contributed to its overall antiviral activity.

To determine if CD4-BFFI has antiviral activity against virus strains resistant to CD4 mAbs we identified several HIV-1 strains which cannot or only partially be inhibited by the CD4 mAb 6314 (Tables 1–3). Partial inhibition is linked to resistance in clinical trials (Jacobson et al., 2008) and natural low susceptibility to CD4 mAbs seems to be a common phenomenon: The CD4 mAbs MEM115 and RPA-T4, for which anti- HIV-1 activity has been described (Hong et al., 1999; Horvath et al., 2005), could not fully inhibit all or one of four virus strains tested (Table 5). In contrast, CD4-BFFI protected PBMC from infection by all four CD4 mAb resistant virus strains (Table 5). This suggests that the dual mechanism of action of CD4-

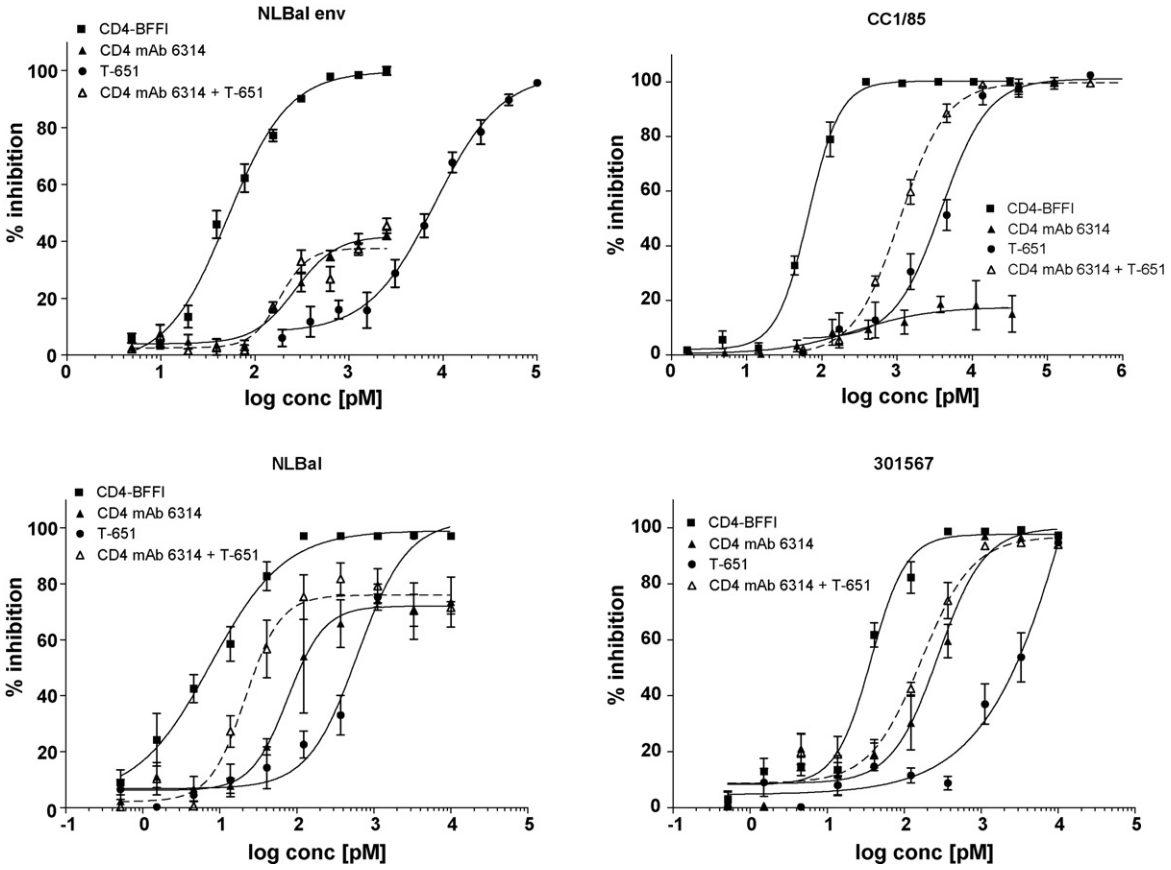

Fig. 3. Increased antiviral potency of CD4-BFFI compared to the fusion inhibitor T-651, CD4 mAb 6314 or a combination of both. (A) JC53BL cells were infected in a single cycle entry assay with HIV-1 particles pseudotyped with R5-tropic NLBal envelope in the presence of CD4-BFFI (■), CD4 mAb 6314 (▲), T-651 (●) or a 2:1 molar combination of T-651 and 6314 (Δ). (B-D): PBMC were infected with the HIV-1 virus strains CC1/85 (B), NLBal (C) or 301567 (D) in the presence of the same inhibitors as in (A). The average and standard deviation of one representative example of three independent experiments are shown.

Table 4
Antiviral potency of the fusion inhibitors T-651, T-20 and T-1144, the CD4 mAb 6314 and CD4-BFFI against HIV-1 strains with resistance to fusion inhibitors.

Virus	Resistance	IC_{50} [ng/ml]				
		T-651	T-20	T-1144	CD4 mAb 6314	CD4-BFFI
3′GIV	Wildtype	6.3 ± 0.6	119.0	n.d.	48% max. inhib. [a]	4.8 ± 1.0
NL178	T-20	1.4 ± 1.1	>10,000 [b]	n.d.	59% max. inhib. [a]	2.0 ± 1.0
DH012	Wildtype	69.9 ± 17.1	1206.0	n.d.	60% max. inhib. [a]	18.1 ± 5.8
DH12RF178	T-20	53.1 ± 11.4	>10,000 [b]	n.d.	60% max. inhib. [a]	11.5 ± 2.8
IIIB	Wildtype	1.8 ± 0.8	74.6	n.d.	45% max. inhib. [a]	3.7 ± 1.5
Re4	T-20	11.3 ± 3.6	>10,000 [b]	n.d.	65% max. inhib. [a]	3.1 ± 1.2
RF	Wildtype	6.5 ± 1.7	115.6	n.d.	88% max. inhib. [a]	2.8 ± 1.3
RFRD178	T-20	8.8 ± 1.4	>10,000 [b]	n.d.	85% max. inhib. [a]	3.3 ± 0.6
098	Wildtype	34.9 ± 12.9	n.d.	32.7 ± 9.6	n.d.	9.6 ± 5.2
098-1144	T-1144	404.9 ± 83.5	n.d.	258.5 ± 0.4	n.d.	19.4 ± 5.5

Shown are the average IC_{50} ± SD from 3 independent experiments for T-651, T-1144, 6314 and 6311. T-20 dose–response curves were performed once in quadruplicates.
[a] The maximal inhibition is shown instead of the IC_{50} in cases were the dose–response curves show a plateau at levels less than 90%.
[b] >10,000: IC_{50} was not reached at assay top concentration of 10,000 ng/ml.

Table 5
Antiviral potency of the CD4-BFFI, the CCR5 antagonist maraviroc and the anti-CCR5 mAb 3952 against HIV-1 isolates resistant to maraviroc or 3952 in the PBMC assay.

Virus	IC_{50} [nM]		
	CD4-BFFI	Maraviroc	CCR5 mAb 3952
CC1/85_NDC	0.07 ± 0.01	0.54 ± 0.12	n.d.
CC1/85_MVRres	0.14 ± 0.03	>20,000[a]	n.d.
Bal_NDC	0.07 ± 0.01	n.d.	1.74 ± 0.09
Bal-3952res	0.05 ± 0.01	n.d.	>100[a]

Shown are the average IC_{50} ± SD from 3 or more independent experiments.
[a] No inhibition: flat dose–response curves; no indication of inhibition at concentrations up to 20 μM or 100 nM, respectively

BFFI can overcome resistance to either one of its components, by compensating with the alternate mechanism of inhibition—either the CD4 mAb or the fusion inhibitor.

Finally, we analyzed if CD4-BFFI is active against virus variants resistant to other entry inhibitors. We recently isolated a virus strain resistant to the CCR5 antagonist maraviroc as well as to two strains resistant to the anti-CCR5 mAb 3952 (Jekle et al., 2008; Jekle et al., 2007). CD4-BFFI inhibited the maraviroc and the CCR5 mAb 3952-resistant virus variants with no or minimal loss of antiviral activity (Table 5 and data not shown).

Taken together, CD4-BFFI is active against HIV-1 variants resistant to entry inhibitors with different mechanism of action such

as CCR5 small molecule antagonists, anti-CCR5 and anti-CD4 antibodies and fusion inhibitors. Furthermore, the dual mechanism of action ensures antiviral activity even in the presence of resistance to one mechanism.

3.4. Mechanism of action of CD4-BFFI

The increased antiviral activity of CD4-BFFI compared to T-651 and 6314 (Fig. 3) suggests that both pharmacophores contribute to its antiviral activity. The potent inhibition of the CC1/85 virus strain, which is largely insensitive against the anti-CD4 mAb 6314, indicates that the fusion inhibitor portion of CD4-BFFI (T-651) plays a major part in the antiviral activity. To further test this hypothesis, we set up a synchronized viral infection experiment that allows determination of the latest possible time of interference during viral entry for different entry inhibitors to take effect. Viral particles were allowed to bind to the cell surface and receptors, but subsequent steps were halted at low temperature. Infection resumed by adding warm media. The anti-CD4 mAb 6314 effectively inhibited viral infection when added at the same time as the warm media or shortly afterwards ($t_{1/2}$ = 18.5 min) as expected for an inhibitor of the earliest step in the HIV-1 entry process (Fig. 4). The CCR5 mAb 3952 could be added slightly later ($t_{1/2}$ = 26.8 min) and still exert a strong antiviral effect. In contrast, the fusion inhibitor T-651, which blocks one of the last steps of HIV-1 entry, was still active when added more than 30 min after the release of the temperature arrest ($t_{1/2}$ = 55.9 min). The time window when CD4-BFFI was active overlapped with the one for T-651 ($t_{1/2}$ = 53.8 min), suggesting that CD4-BFFI can act during the fusion step (Fig. 4). However, since this experiment allows only the determination of the latest possible time of action, it does not exclude the possibility that the CD4 mAb portion acts during the earlier step of CD4 engagement.

Finally, we investigated the contribution of the anti-CD4 mAb portion of CD4-BFFI to its overall antiviral potency and/or whether its major role is to anchor the relatively large molecule at the cell surface and thereby increase the local concentration of the fusion inhibitor at the site of action. We have shown previously for the CCR5 mAb based BFFI that the anti-CCR5 mAb portion of this molecule acts as a membrane anchor (Kopetzki et al., 2008). The molecule was inactive in protecting cells that did not express CCR5 against X4-tropic HIV-1 infection (Kopetzki et al., 2008). To determine if the CD4 mAb part of CD4-BFFI has a similar membrane-anchoring function, we preincubated PBMC with

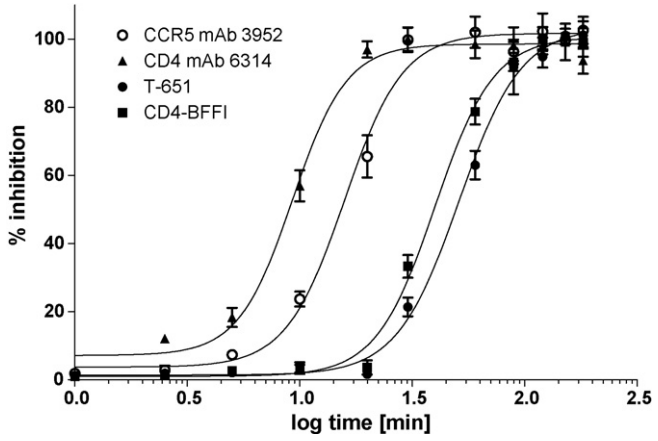

Fig. 4. Synchronized infections studies: The HIV-1 strain RF was bound to JC53BL cells by spin-infection for 2 h at 4 °C. The temperature arrest was released by addition of 37 °C warm media. CCR5 mAb 3952 (○), CD4 mAb 6314 (▲), T-651 (●) or CD4-BFFI (■) were added at the indicated times at a final concentration of 5 μg/ml. The averages and standard deviation of one representative example of three independent experiments carried out in quadruplicates are shown.

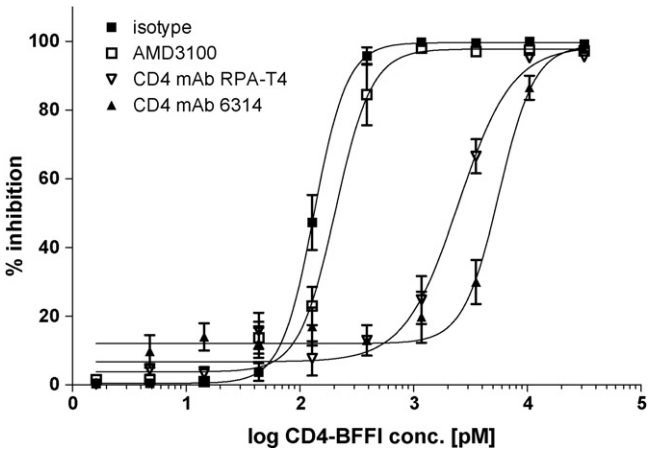

Fig. 5. Pre-incubation with CD4 mAbs reduces the antiviral potency of CD4-BFFI: PBMC were pre-incubated with 5 μM AMD3100 (□) or 5 μg/ml of the CD4 mAbs RPA-T4 (▽) or 6314 (▲) or an isotype control antibody (■) for 60 min at 37 °C. Cells were washed and infected with the HIV-1 strain CC1/85 in presence of CD4-BFFI. The averages and standard deviation of one representative example of three independent experiments carried out in quadruplicates are shown.

5 μg/ml of the CD4 mAbs 6314 or RPA-T4, an isotype control antibody or 5 μM of the CXCR4 inhibitor AMD3100 for 60 min. Following the pre-incubation, we infected the cells with the R5-tropic virus CC1/85 in presence of serially diluted CD4-BFFI. We showed above that neither 6314 nor RPA-T4 significantly inhibit the R5-tropic virus CC1/85 (Table 5). Pre-incubation with the isotype control or AMD3100 did not interfere with inhibition of viral infection by CD4-BFFI (Fig. 5). In contrast, pre-incubation with the CD4 mAbs 6314 and RPA-T4 reduced the antiviral potency of CD4-BFFI 19- to 42-fold compared to the isotype control (Fig. 5). This suggests that the pre-binding of the CD4 mAbs reduced binding of CD4-BFFI to the target cells, thereby decreasing the local concentration of the fusion inhibitor on the cell surface and reducing inhibition of infection.

4. Discussion

Here we describe a novel, bifunctional entry inhibitor for the treatment of HIV/AIDS. CD4-BFFI is composed of an anti-CD4 mAb linked to two copies of a fusion inhibitor. The concept is based on our earlier bifunctional fusion inhibitor BFFI, which consisted of an anti-CCR5 mAb linked to a fusion inhibitor (Kopetzki et al., 2008). BFFI has increased antiviral potency as long as the target cells express CCR5. We explained this observation with an anchoring model, according to which, BFFI has to bind via its CCR5 mAb portion to CCR5 receptors on the cell surface to bring the fusion-inhibitor portion to the site of action. Without anchoring of the molecule to the cell surface the high molecular weight of BFFI hampers diffusion and access of the fusion inhibitor portion to the 6-helix bundle formation during the fusion process. Therefore, it can only protect cells that do express CCR5 against X4-tropic HIV-1 infection. Based on this hypothesis, we now designed a novel bifunctional HIV-1 entry inhibitor with high and broad antiviral potency against all tested HIV-1 strains independent of their co-receptor preference and target cells. Taking advantage of the essential role of the CD4 receptor during HIV-1 entry we used an anti-CD4 mAb to anchor the molecule to the surface of all target cells.

The bi-functionality of CD4-BFFI brings two advantages: an increase in antiviral potency due to two independent mechanisms of actions and retained antiviral potency against entry inhibitor-resistant virus strains. We provide several lines of evidence to demonstrate that both portions of CD4-BFFI contribute to its overall antiviral activity. Especially on a molar basis, CD4-BFFI is more

Table 6
Antiviral potency of the CD4 mAbs 6314, MEM115 and RPA-T4 and CD4-BFFI against selected HIV-1 strains in the PBMC assay.

Virus	Tropism	IC_{50} [ng/ml]			
		CD4 mAb 6314	CD4 mAb MEM115	CD4 mAb RPA-T4	CD4-BFFI
NLBal	R5	61% max. inhib.[a]	31% max. inhib.[a]	68.3 ± 39.2	2.9 ± 1.8
CC1/85	R5	20% max. inhib.[a]	no inhibition[b]	no inhibition[b]	14.9 ± 6.7
301567	R5	18.0 ± 14.2	no inhibition[b]	30.1 ± 18.4	11.5 ± 9.0
89.6	R5X4	19.2 ± 2.5	no inhibition[b]	8.35 ± 3.9	10.2 ± 5.5

Shown are the average IC_{50} ± SD from 3 or more independent experiments.
[a] The maximal inhibition is shown instead of the IC_{50} in cases were the dose–response curves plateau at levels less than 90%.
[b] No inhibition: flat dose–response curves; no indication of inhibition at concentrations up to 5 μM.

active than either the fusion inhibitor T-651 or the CD4 mAb 6314 alone or in combination (Tables 1–3, Fig. 3) suggesting that both pharmacophores of CD4-BFFI have independent antiviral activity. More importantly, CD4-BFFI can fully inhibit every virus tested while the CD4 mAb 6314 demonstrated only partial dose–response curves against many virus strains. This implies that these naturally 6314-resistant virus strains are still susceptible to the fusion inhibitor portion of CD4-BFFI. Conversely, while T-651 has a 12-fold reduced antiviral activity against the partially T-1144-resistant virus variant 098-1144, the CD4-BFFI activity is reduced only two-fold indicating that the CD4 mAb portion can compensate for the loss of activity of the fusion inhibitor portion.

Furthermore, CD4-BFFI was active against HIV-1 variants with resistance to different classes of entry-inhibitors such as the fusion-inhibitors T-20 and T-1144, the CCR5 antagonist maraviroc and the CCR5 mAb 3952. While we did not perform *in vitro* resistance development experiments, we hope that the dual mode of action and the antiviral activity of CD4-BFFI against the entry-inhibitor resistant strains translates into a high barrier to resistance. It has already been described that T-651 has a high resilience against resistance development (Dwyer et al., 2007). The additional antiviral activity of the CD4 mAb should further increase this barrier to resistance. This is supported by the observed antiviral activity of CD4-BFFI against the 098-1144 virus variant (Table 4). More striking is the increased durability of CD4-BFFI compared to the CD4 mAb 6314 or other CD4 mAbs tested. Many virus strains are only partially inhibited by 6314 but were completely inhibited by CD4-BFFI (Tables 1–4 and 6). Partial inhibition has been associated with virological failure in the clinic (Jacobson et al., 2008) and has been described earlier for allosteric inhibitors such as CCR5 inhibitors (Pugach et al., 2007; Westby et al., 2007). Ibalizumab and the Ibalizumab-derived antibody 6314 do not compete with binding of HIV gp120 to the D1 domain of CD4 but interfere with a subsequent step by binding to its D2 domain (Burkly et al., 1992). It is therefore conceivable that the incomplete inhibition of many virus strains by 6314 is a consequence of the virus having acquired the ability to use the CD4 receptor in an antibody bound state. Data from a recent Ibalizumab clinical trial showed that 93% of virus trains isolated after nine weeks of monotherapy could no longer be fully inhibited by Ibalizumab indicating that HIV might develop resistance to allosteric CD4 antibodies relatively quickly and frequently. CD4-BFFI with its dual mechanism of inhibition should be able to prevent fast arising resistance development.

The antiviral activity of entry inhibitors such as CD4-BFFI might depend on the numbers of receptors expressed on the target cells. In this study, we used activated PBMC and JC53BL cells, which over-express the HIV receptors CD4, CXCR4 and CD4 to determine the antiviral potency of CD4-BFFI and its components 6314 and T-651. We previously quantified the number of CD4, CCR5 and CXCR4 receptors on these cells (Ji et al., 2009). The antiviral activity of CD4-BFFI on these cell lines falls within a narrow range independent of the cell type, high (JC53BL) or low (PBMC) CD4 surface expression or weather replication-competent viruses (Tables 2 and 3) or pseudotyped viral particles (Table 1) were used. The antiviral activity of CD4-BFFI was not specifically measured in monocytes/macrophages. Given the fact that the numbers of CCR5, CXCR4 and CD4 receptors expressed on monocytes and macrophages are within the same range as what we observed on PBMC and JC53BL cells, we expect that the antiviral activity of CD4-BFFI will also be similar to what we observed in the other cell types (Ji et al., 2009; Lee et al., 1999).

The antiviral activity of CD4-BFFI was analyzed in this study using more than 40 different virus strains. CD4-BFFI had high antiviral activity with low picomolar IC_{50} against all of these strains independent of co-receptor usage or clade affiliation. We could also not detect any difference in antiviral potency weather lab-adapted virus strains (NLBal, JRFL, SF162, NL4-3 and HXB2 in Table 1 and JRCSF, NLBal, Bal, 89.6 and NL4-3 in Table 3) or minimally expanded clinical isolates (W969-5#1, 93TH6.9, 2148#58, 1013#3, CS81-69-E09, CS81-4-G0, C597-8-H0 in Table 1 and RU570, 301567, 92TH599, BK132 and BZ162 in Table 3) were used. Finally, it appears that CD4-BFFI is less active than T-651 against NL4-3 in the single cycle entry assay (Table 1), however on a molar basis it is 25-fold more active. Furthermore, in the biologically more relevant PBMC assay, CD4-BFFI is more than 1200-fold more active against NL4-3 than T-651 on a molar basis.

The current treatment of AIDS patients is a combination of three or more antiretroviral medicines called HAART (highly active antiretroviral therapy). Common challenges to HAART are high pill burden resulting in poor adherence, resistance development, drug-induced toxicities and drug–drug interactions. The bifunctionality of the CD4-BFFI molecule described here has the potential to address several of these challenges: (1) the combination of two pharmacophores in one molecule has the potential to reduce the number of medicines needed in the cocktail. Adherence can be improved by infrequent dosing regimens and the long plasma half-life of antibody-based antiviral drugs could allow biweekly or monthly administration (Jacobson et al., 2004; Kuritzkes et al., 2004; Lalezari et al., 2008). (2) An entry inhibitor with dual mechanism of action should increase the genetic barrier for resistance development, since both pharmacophores can act independently and resistance to both pharmacophores should be rare and might affect viral fitness. CD4-BFFI is active against virus strains resistant to entry inhibitors with different modes of action such as fusion inhibitors, CCR5 mAbs and CCR5 antagonists (Table 5). (3) A general advantage of antibody-based therapeutics over small molecule compounds is their high target specificity which minimizes the non-target associated side effects and toxicities. In *in vitro* experiments, CD4-BFFI as well as the anti-CD4 mAb 6314 and T-651 had no apparent cytotoxicity at concentrations up to 50 μg/ml, resulting in therapeutic indices of more than 5000 for CD4-BFFI and more than 400 for T-651 and 6314 (Fig. 2). Furthermore, CD4-BFFI and 6314 did not cause or alter antigen-specific and -unspecific T-cell activation and cytokine release (unpublished data). Treatment of three cynomolgus monkeys with 10 mg of CD4-BFFI/kg of body weight was well-tolerated and did not cause apparent CD4-depletion or

stimulation of CD4 T-cells (Ji et al., 2009) (and unpublished data). Ibalizumab, to which 6314 is closely related, was also well tolerated in clinical studies and did not show any CD4-depletion (Jacobson et al., 2008). Similarly, the fusion inhibitor enfuvirtide is safe and with the exception of injection-site reactions well tolerated (Trottier et al., 2005). We therefore expect that a combination of an antibody with a peptidic fusion inhibitor should also have a favorable safety profile. (4) Finally, antibodies have a different route of administration, metabolism and clearance, reducing the risk of drug–drug interactions with small molecules.

An obvious disadvantage of protein-based therapeutics is their need of parenteral administration while the market for antiretroviral drugs clearly favors oral delivery. Replacing antibodies and peptides with small molecule drugs is desirable but would come with the cost of lower target specificity probably causing increased toxicity, a lower therapeutic index and the risk of drug–drug interactions with other medicines.

The risk of anti-drug antibody (ADA) development is another challenge that protein therapeutics such as CD4-BFFI may face. We tried to reduce this risk by using (1) a humanized antibody scaffold; (2) a fusion inhibitor based on a natural HIV sequence; (3) choosing a linker sequence that according to *in silico* prediction tools is not immunogenic. Ultimately, the extent of ADA formation and its consequence on the antiviral potency of CD4-BFFI have to be determined in clinical studies.

To reach clinical efficacy, viral entry inhibitors have to completely occupy all target receptors. CD4 is expressed on many lymphocyte subsets, monocyte/macrophages and dendritic cells. Data from a small pharmacokinetic (PK) study in cynomolgus monkeys suggest that with a single dose of 10 mg CD4-BFFI per kg bodyweight 100% receptor occupancy can be reached and sustained for seven days (Ji et al., 2009). While an accurate prediction of human PK properties of CD4-BFFI based on the limited monkey PK study cannot be made, these data are in line with results from clinical trials studying the human CD4 antibody Ibalizumab (Jacobson et al., 2008).

In conclusion, CD4-BFFI is a novel bifunctional HIV-1 entry inhibitor with high antiviral potency against virus strains with different cellular tropism, genetic background and clade classification. CD4-BFFI inhibits HIV-1 variants resistant to several classes of entry inhibitors. Its bifunctionality and antibody scaffold suggests an advantageous clinical profile with high and broad antiviral potency and low risk of side effects or drug–drug interactions.

Acknowledgements

We would like to thank Dr. Shaw (University of Alabama, Birmingham), Dr. Chen (University of California, Los Angeles), Dr. Stoddart (The J. David Gladstone Institutes, San Francisco, California), Dr. Richman (University of California, San Diego) and Dr. Greenberg (Trimeris, Morrisville, NC) for valuable reagents. Thanks to Nixy Zutshi and Saundra Clausen at Roche Palo Alto for their help with cell culture and to Sophie Chiu for statistical analysis. Special thanks to Milloni Chhabra for her assistance in the preparation of the manuscript.

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