



ADS-J1 inhibits HIV-1 infection and membrane fusion by targeting the highly conserved pocket in the gp41 NHR-trimer



Fei Yu ^{a,b,c}, Lu Lu ^b, Qi Liu ^b, Xiaowen Yu ^c, Lili Wang ^c, Elaine He ^c, Peng Zou ^c, Lanying Du ^c, Rogier W. Sanders ^{d,e}, Shuwen Liu ^{a,*}, Shibo Jiang ^{a,b,c,**}

^a School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China

^b Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College, Fudan University, Shanghai 200032, China

^c Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10065, USA

^d Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

^e Weill Medical College of Cornell University, Department of Microbiology and Immunology, New York, NY 10065, USA

ARTICLE INFO

Article history:

Received 15 October 2013

Received in revised form 24 December 2013

Accepted 27 December 2013

Available online 3 January 2014

Keywords:

HIV-1

gp41

Pocket

Six-helix bundle

Mutation

HIV fusion inhibitor

ABSTRACT

We previously identified a potent small-molecule human immunodeficiency virus type 1 (HIV-1) fusion inhibitor, termed ADS-J1, and hypothesized that it mainly targeted the hydrophobic pocket in the gp41 N-terminal heptad repeat (NHR) trimer. However, this hypothesis has been challenged by the fact that ADS-J1 cannot induce drug-resistance mutation in the gp41 pocket region. Therefore, we show herein that HIV-1 mutants resistant to T2635, a peptide derived from the gp41 C-terminal heptad repeat (CHR) region with pocket-binding domain (PBD), were also resistant to ADS-J1. We also show that pseudoviruses with mutations at positions 64 and 67 in the gp41 pocket region were highly resistant to ADS-J1 and C34, another CHR-peptide with PBD, but relatively sensitive to T20, a CHR-peptide without PBD. ADS-J1 could effectively bind to N36Fd, a mimic of the gp41 NHR-trimer with pocket exposed, and block binding of C34 to N36Fd trimer to form six-helix bundle (6-HB). However, ADS-J1 was less effective in binding to N36Fd trimer with mutations in the gp41 pocket region, such as N36(Q64A)Fd, N36(Q64L)Fd, N36(A67G)Fd, N36(A67S)Fd, and N36(Q66R)Fd, as well as less effective in blocking 6-HB formation between C34 and these mutant N36Fd trimers. These results confirm that ADS-J1 mainly targets the pocket region in the HIV-1 gp41 NHR trimer and suggest that it could be used as a lead for developing small-molecule HIV fusion inhibitors and as a molecule probe for studying the mechanisms of gp41-mediated membrane fusion.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Gp41 is the transmembrane subunit of the envelope glycoprotein (Env) of the human immunodeficiency virus type 1 (HIV-1). As such, it plays a key role in HIV-fusion with, as well as entry into the host cell, thus serving as an attractive target for developing an HIV-1 fusion inhibitor. In the early 1990s, we and others identified several potent anti-HIV-1 peptides derived from the HIV-1 gp41 C-terminal heptad repeat (CHR), such as SJ-2176 [18], DP178 (also known as T20) [41]

Abbreviations: CD, Circular Dichroism; CHR, C-terminal heptad repeat; Env, envelope protein; Fd, foldon; HIV-1, human immunodeficiency virus 1; IC₅₀, concentration causing 50% inhibition; NHR, N-terminal heptad repeat; N-PAGE, native polyacrylamide gel electrophoresis; PBD, pocket-binding domain; 6-HB, six-helix bundle

* Corresponding author.

** Correspondence to: S. Jiang, Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College and Institute of Medical Microbiology, Fudan University, Shanghai 200032, China.

E-mail addresses: liusw@smu.edu.cn (S. Liu), shibojiang@fudan.edu.cn (S. Jiang).

and C34 [31,32] (Fig. 1). T20 (brand name, Fuzeon; generic name, enfuvirtide) was approved by the U.S. FDA as the first HIV fusion inhibitor for treatment of HIV/AIDS patients who fail to respond to the current antiretroviral drugs. However, its clinical application is limited because of its poor efficacy and bioavailability necessitating intramuscular injection twice daily and easy induction of drug resistance [28].

Previous studies have demonstrated that the highly conserved hydrophobic pocket in the groove of the gp41 N-terminal heptad repeat (NHR) trimer plays a critical role in stabilizing the interaction between the gp41 NHR and CHR, formation of the six-helix bundle (6-HB) core, and HIV-1 fusion with the target cell, thereby serving as an important target for development of HIV-1 fusion inhibitors [5,17,34]. The CHR-peptides with the pocket-binding domain (PBD), such as C34, T2635, T1144 and CP32M, have much more potent HIV-1 fusion inhibitory activity and much higher barrier to drug resistance than the CHR-peptides without the PBD, such as T20 [10,12,15,26,27].

The GIV motif (residues 36–45: GIVQQNLL) in the N-terminal region of the HIV-1 gp41 NHR is a major determinant of HIV-1 resistance

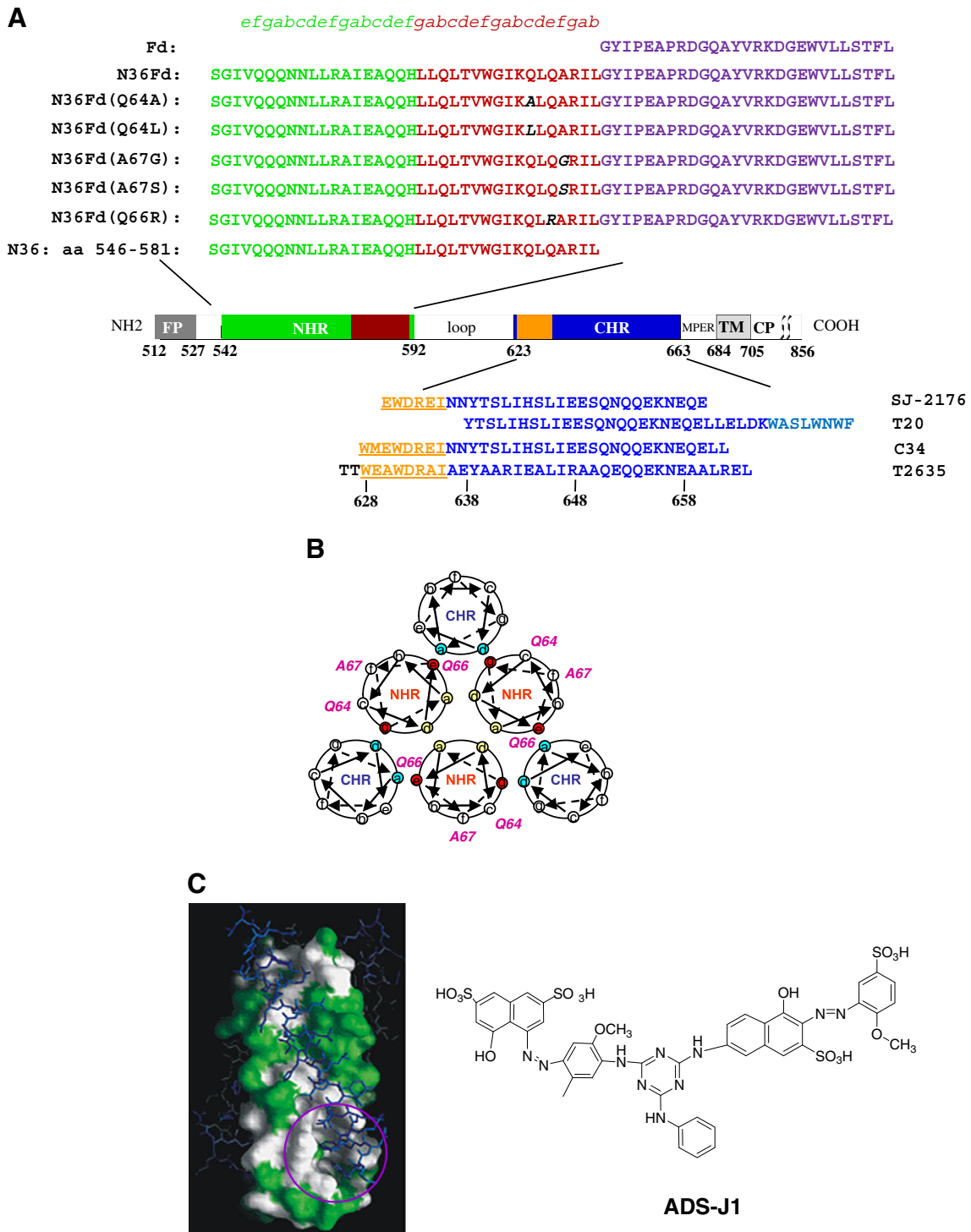


Fig. 1. The HIV-1 gp41 functional domains and the structure of ADS-J1. (A) HIV-1 gp41 and corresponding N- and C-peptide sequences. The residue number corresponds to its position in HIV-1_{HXB2} gp160. FP, fusion peptide; NHR, N-terminal heptad repeat (in green), which contains a pocket-forming domain (in brown); CHR, C-terminal heptad repeat (in blue), which contains a pocket-binding domain (in orange); MPER, membrane proximal external region; TM, transmembrane domain; CP, cytoplasmic domain. The amino acid sequences of N-peptides, and the mutants of N-peptides and C-peptides are shown. (B) Schematic view of the HIV-1 gp41 6-HB core. The letters “a–g” indicate the positions of the corresponding residues in the helical wheel of the gp41 NHR domain. Residues Q64, Q66, and A67 are located at the c, e, f sites, respectively. Residue Q66 involves direct interaction with the key residues in NHR for forming N-trimer or those in CHR for forming 6-HB, but not Q64 and A67. The 6-HB is formed through the interaction of residues located at the e and g positions (in red) in the N helices and those at a and d positions (in green). (C) The schematic representation of the 6-HB (left panel, adapted from ref. 4) and the chemical structure of ADS-J1 contains four sulfonic acid groups (right panel). The hydrophobic pocket in the groove of NHR-trimer is highlighted by a purple circle. One of ADS-J1’s sulfonic acid groups is expected to interact with the basic residue K574 in the pocket-forming sequence in NHR.

to CHR-peptides with the GIV-binding domain. Since the major binding site of T20 is located in this region, HIV-1 variants with mutations in the GIV motif isolated from T20-treated patients or T20-containing cell cultures were found to be highly resistant to T20 [4,23,33,36,39,42]. On the

other hand, CHR-peptides without GIV-binding domain, such as CP32M, proved highly effective against the T20-resistant strains [15].

We previously identified the first small-molecule HIV fusion inhibitor, termed ADS-J1 (Fig. 1), with computer-aided virtual screening using

the gp41 pocket as a target and the gp41 6-HB inhibition assay [9]. We found that ADS-J1 could directly bind to the gp41 pocket presented on the IQN17 trimer, block the binding of a short D peptide, PIE7 [11], to the gp41 pocket, and inhibit 6-HB formation between NHR- and CHR-peptides [9,38]. Moreover, ADS-J1 could effectively inhibit HIV-1 Env-mediated membrane fusion and HIV-1 replication [9,38]. Therefore, we proposed ADS-J1 as an HIV fusion inhibitor that mainly targets the conserved hydrophobic pocket on the gp41 NHR-trimer. The finding was, however, challenged by Este and colleagues, who concluded that ADS-J1 does not target the gp41 pocket, but rather the gp120, particularly the V3 loop. This conclusion was based on evidence that ADS-J1 could induce resistant mutations in the highly variable V3 loop region in gp120, but not the gp41 pocket region [3,14].

To rebut this finding and test our hypothesis that the main target site of ADS-J1 is, in fact, the pocket region in the HIV-1 gp41 NHR-trimer, we herein assessed the sensitivity to ADS-J1 of HIV-1 strains with mutations in the gp41 pocket and the resistant mutants induced by T2635, a CHR-peptide with PBD [12]. We found that T2635-resistant HIV-1 mutants were also resistant to ADS-J1 and that their resistance to T2635 and ADS-J1 was correlated, presumably because they may mainly target the same site, i.e., the pocket region in gp41. It was additionally found that pseudotyped HIV-1 strains with mutations in the gp41 pocket region (Q64A, Q64L, A67G, A67S, and Q66R) were highly resistant to ADS-J1 and C34, but relatively sensitive to T20. These results suggest that similar to the CHR-peptides with PBD, such as T2635 and C34, ADS-J1 inhibits HIV fusion by mainly targeting the pocket region of the HIV-1 gp41 NHR-trimer. Therefore, ADS-J1 can be used as a molecule probe for studying the mechanisms of gp41-mediated membrane fusion and as a lead for developing small-molecule HIV fusion inhibitors.

2. Materials and methods

2.1. Preparation and titration of HIV Env-pseudoviruses

HIV-1 pseudoviruses with wild-type and mutated Env were generated as previously described [30,43]. In brief, the mutations (Q64A, Q64L, A67G, and A67S) were introduced into the pHXB2-Env by using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The plasmid encoding wild-type or mutant HXB2-Env was co-transfected with a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.Luc.R-E-) into HEK293T cells using FuGENE 6 reagents (Roche Applied Science). The supernatants, which contain the pseudovirions, were collected 48 h post-transfection, centrifuged at 1000 ×g for 10 min, and filtrated through a 0.45 μm filter to remove cells. The virions in the supernatants were concentrated by centrifuging at 26,000 ×g for 2 h, and the concentrations of these pseudovirion stocks were determined by enzyme-linked immunosorbent assay (ELISA) for p24 antigen and stored at −80 °C before use.

2.2. Propagation of T2635-resistant HIV-1 variants

The plasmids of one wild-type (HIV-1 LAI molecular clone) and thirteen T2635-resistant HIV-1 clones were constructed and propagated as described before [12]. Briefly, each plasmid (20 μg) was transfected into HEK293T cells in a six-well plate by using the calcium phosphate method. Supernatants containing T2635-resistant viruses were harvested 72 h post-transfection. Then the virions in supernatants were further expanded by infecting MT-2 cells. Cytopathic effect (CPE) was observed, and the supernatants were collected at days 4, 5, 6, and 7, respectively. The virions in the supernatants at peak production time were harvested, filtered, concentrated, titrated as described above, and stored at −80 °C before use.

2.3. Determination of infectivity of HIV-1 pseudovirus and HIV-1 LAI clones

An HIV-1 pseudovirus and its mutants (250 ng p24/ml) or HIV-1 LAI clone and its T2635-resistant mutants (5 ng p24/ml) were preincubated

with a test compound at indicated concentration at 37 °C for 30 min. The mixture was added to TZM-BL cells (1×10^4 /well), followed by incubation at 37 °C for 12 h. The culture medium was replaced with fresh DMEM. After culture for an additional 48 h, the medium was removed. The cells were washed with PBS and lysed using the lysis reagent from a luciferase kit (Promega, Madison, WI). The cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar, Corning, NY) and luciferase substrate (Promega) was added to the plate, followed by immediate measuring for luciferase activity (relative light units, RLU) using an Ultra 384 luminometer (Tecan, USA).

2.4. Construction of vectors encoding N36Fd trimer and its mutants

To construct vectors encoding N36Fd and its mutants, the corresponding DNA fragments in the pseudovirus mutants Q64 (A/L) and A67 (G/S), as well as the T2635-resistant virus carrying a Q66R mutation, were amplified by PCR using the Platinum PCR SuperMix High Fidelity Kit (Invitrogen) with a forward primer containing a BamH1 site and a reverse primer coding the first 6 amino acids of the Fd fragment. The DNA fragment of Fd was amplified by PCR using a forward primer (the first 17 amino acids of Fd) and a reverse primer (the last 17 amino acids of Fd). Then the two overlapping fragments were mixed and used as templates for another PCR reaction using the BamH1 forward primer and a shorter reverse primer coding the last 8 amino acids of the Fd with an Xho1 site. The product was purified using a gel extraction kit (Qiagen, Valencia, CA), digested with BamH1 and Xho1 enzymes (TaKaRa Bio, Madison, WI), and cloned into a pGEX6p-1 vector (Qiagen). The sequence was confirmed by DNA sequencing.

2.5. Protein expression and purification

N36Fd-trimer-pGEX6p-1 plasmids encoding N36Fd trimer and its mutants were transformed into *Escherichia coli* Rosetta 2 (DE3) (Novagen, Gibbstown, NJ). The cells were incubated at 37 °C in LB medium until the A600 reached 0.8–1.0. The culture was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside and incubated at 18 °C for 20 h. Then, the cells were harvested and broken by sonication in 1% Triton PBS buffer. After the samples were centrifuged, the supernatant was loaded into a GST-Bind column (Novagen). The column was rinsed and the bound GST-fused N36Fd and its mutants were then cleaved with PreScission Protease (GE Healthcare) in cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol) at 4 °C overnight. The cleaved N-trimer was eluted from the column on the next day by washing with cleavage buffer. The rough product was further separated from GST by a series of ultrafiltrations using Amicon Ultra-15 Centrifugal Filter Devices (Millipore, Billerica, MA). N36Fd and its mutants became monomers in the buffer with pH lower than 3.0 and were able to pass through the 30 kDa Ultra-15 Centrifugal Filter Device and be collected, while GST that remained in the filter unit was discarded. After dialysis against ddH₂O (pH 7.0), the monomeric N36Fd and its mutants were refolded into trimers and concentrated by using a 3 kDa Ultra-15 Centrifugal Filter Device.

2.6. Native polyacrylamide gel electrophoresis (N-PAGE)

N-PAGE was used to detect the inhibitory activity of ADS-J1 on formation of 6-HB between a C-peptide and an N-trimer or its mutant as described previously [29]. Briefly, the mixture of an N36Fd trimer, or its mutant (40 μM), and ADS-J1 at an indicated concentration was incubated at 37 °C for 30 min, followed by addition of the C-peptide C34 (40 μM). After incubation at 37 °C for 30 min, the mixture was loaded onto the 18% Tris-glycine gel (Invitrogen). Gel electrophoresis was carried out at 125 V constant voltage at room temperature for 2 h. The gel was stained with Coomassie Blue and imaged with a FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA).

2.7. CD spectroscopy

The secondary structure of N36Fd trimer, or its mutants and resultant complexes, with C34 peptide in PBS (pH 7.2) were determined by CD spectroscopy as previously described [8,29]. Briefly, the N36Fd trimer, or its mutants (10 μ M), were incubated with PBS or ADS-J1 (50 μ M) at 37 °C for 30 min, followed by addition of C34 (10 μ M). After further incubation at 37 °C for 30 min, the samples were cooled to room temperature. The spectra of each sample were acquired on a spectropolarimeter (Model J-715, Jasco Inc., Japan) at room temperature, using a 5.0 nm bandwidth, 0.1 nm resolution, 0.1 cm path length, 4.0 s response time, and 50 nm/min scanning speed. The spectra were then corrected by subtraction of a background corresponding to the solvent. A $[\theta]_{222}$ value of $-33,000 \text{ deg cm}^2 \text{ dmol}^{-1}$ was taken to correspond to 100% α -helical content as described previously.

2.8. Isothermal titration calorimetry (ITC)

Binding of N36Fd, or its mutant, to ADS-J1 was measured using an isothermal titration calorimetry instrument (NANO ITC, TA Instruments, Lindon, UT), which directly measures the heat released or absorbed during the binding process. ADS-J1 (750 μ M) dissolved in PBS (pH 7.2) was injected into the ITC cell containing 50 μ M N36Fd, or its mutant (pH 7.2). The experiments were carried out at 37 °C. Data acquisition and analysis were performed using Launch NanoAnalyze software.

3. Results

3.1. T2635-resistant HIV-1 mutants were resistant to T2635 and ADS-J1, but sensitive to AZT

T2635, a 38-mer CHR peptide with PBD, is a highly potent HIV-1 fusion inhibitor by targeting the gp41 pocket region [10]. This peptide is highly effective against T20-resistant strains [12]. After 6 months of culturing T2635 with HIV-1 LAI molecular clone, a series of variants with single, double and multiple mutations in gp41 were identified to be resistant to T2635, suggesting that T2635 resistance is not caused by mutations in the pocket region, but rather in other regions of gp41 that may alter the kinetics of 6-HB formation, thus limiting the time window for T2635 to inhibit membrane fusion [12]. Here, we selected 7, 4, and 2 variants with single, double and multiple mutations, respectively, to compare their sensitivity to T2635, ADS-J1, and AZT (an HIV-1 reverse transcriptase inhibitor as a control) with the wild-type (WT) HIV-1 LAI strain. As shown in Table 1, all the variants with single mutations exhibited moderate resistance to T2635 (4- to 7-fold), while the

four variants with double mutations had higher resistance (13- to 23-fold) than those with single mutation. Those with multiple mutations were highly resistant to T2635 (36- to 263-fold), which are consistent with the report by Sanders and colleagues [12]. Similarly, the variants with single, double and multiple mutations exhibited low, middle and high resistance to ADS-J1, respectively (Table 1 and Fig. 2A). According to the fold of resistance as shown in Table 1, resistance of viruses with single, double and multiple mutations against T2635 is closely correlated with their resistance against ADS-J1 ($r = 0.895$, $P < 0.01$; Fig. 2B). However, these mutants showed sensitivity to AZT similar to that of WT to AZT (Table 1). These results suggest that ADS-J1 and T2635 may share a similar mechanism of action, as well as the same target site in gp41. Mutations in gp41 may alter the kinetics of 6-HB formation, which, in turn, would shorten the time window for T2635 [12] and ADS-J1 to interfere with HIV-1 gp41-mediated membrane fusion.

3.2. Pseudotyped HIV-1 viruses with mutations of Q64 and A67 in the pocket region of the gp41 NHR domain were resistant to ADS-J1 and C34, but relatively sensitive to T20

The HIV-1 pseudoviruses with mutations at residues Q64 and A67 in the pocket region of the gp41 NHR domain, both of which are located in the outer face of the 6-HB (Fig. 1B) and mutations of these residues are not expected to significantly affect the interaction between the viral gp41 NHR and CHR, were constructed as previously described [30,43]. The inhibitory activity of ADS-J1 on infection by the pseudoviruses with wild-type Env sequence (WT) and mutations in gp41 (Q64A, Q64L, A67G, A67S) was determined. As shown in Fig. 3 and Table 2, all the mutant pseudoviruses were highly resistant to ADS-J1 (30- to 91-fold increase of IC_{50} value) and to the PBD-containing CHR peptide C34 (102- to 210-fold increase of IC_{50} value), while they were relatively sensitive to the CHR peptide T20 without PBD (1- to 5-fold increase of IC_{50} value). Similar to C34, these results suggest that ADS-J1 may mainly target the pocket region in the HIV-1 gp41 NHR-trimer.

3.3. Decreased inhibitory activity of ADS-J1 on 6-HB core formation between C34 and N36Fd trimer with mutations in gp41 pocket region

The formation of the 6-HB fusion core is a critical step in gp41-mediated membrane fusion. Using ELISA and N-PAGE, we have previously demonstrated that ADS-J1 can block the 6-HB core formation between N36 and C34 peptide [19,24,29,38]. Here we tested the inhibitory activity of ADS-J1 at graded concentration on the 6-HB formation between C34 peptide and N36Fd trimer, or its mutants, by N-PAGE analysis. As shown in Fig. 4A, C34 could form 6-HB with N36Fd trimer and its

Table 1
Inhibitory activity of T2635 peptide, ADS-J1 and AZT on infection by T2635-resistant HIV-1 variants.

Virus	T2635		ADS-J1		AZT	
	IC_{50} (nM)	Resistance (n-fold)	IC_{50} (nM)	Resistance (n-fold)	IC_{50} (nM)	Resistance (n-fold)
WT	0.4 \pm 0.1	1.0	16.6 \pm 2.2	1.0	15.1 \pm 3.4	1.0
A6V	1.9 \pm 0.9	4.8	59.4 \pm 6.3	3.6	8.4 \pm 0.6	0.6
Q66R	1.9 \pm 0.1	4.7	96.5 \pm 5.1	5.8	9.2 \pm 6.4	0.6
Q79E	1.6 \pm 0.3	4.0	42.5 \pm 14.9	2.6	10.3 \pm 0.5	0.7
K90E	2.6 \pm 1.4	6.5	52.1 \pm 4.6	3.1	11.8 \pm 2.1	0.8
N113E	1.6 \pm 0.2	4.2	54.2 \pm 1.6	3.3	7.1 \pm 2.4	0.5
N126K	2.7 \pm 1.1	6.9	73.4 \pm 4.9	4.4	12.8 \pm 1.6	0.8
K154Q	1.5 \pm 0.2	3.9	89.8 \pm 1.8	5.4	13.6 \pm 0.7	0.9
Q66R N126K	8.6 \pm 0.4	21.9	147.3 \pm 29.5	8.9	13.6 \pm 2.7	0.9
Q79E N126K	5.2 \pm 0.9	13.2	112.1 \pm 9.1	6.8	7.5 \pm 0.8	0.5
K90E N126K	7.5 \pm 0.9	19.3	108.9 \pm 0.6	6.6	6.9 \pm 2.0	0.5
Q66R N113E	8.8 \pm 4.3	22.5	120.4 \pm 33.5	7.3	9.1 \pm 7.2	0.6
H3C	102.7 \pm 46.0	262.7	555.2 \pm 43.9	33.5	39.0 \pm 4.6	2.6
H29C	14.2 \pm 1.3	36.3	184.7 \pm 6.5	11.1	7.8 \pm 1.7	0.5

IC_{50} values are presented in nM. Results are derived from experiments performed in duplicate and are representative of at least three independent experiments. Fold resistance is shown compared to the IC_{50} of the WT (HIV-1 LAI clone). H3C is a T2635-resistant HIV-1 variant with multiple mutations in gp41, including A6V L33S Q66R N126K H132Q E136G; H29C is another T2635-resistant HIV-1 variant with multiple mutations in gp41, including L33S Q66R N113D N125S N126K H132Q.

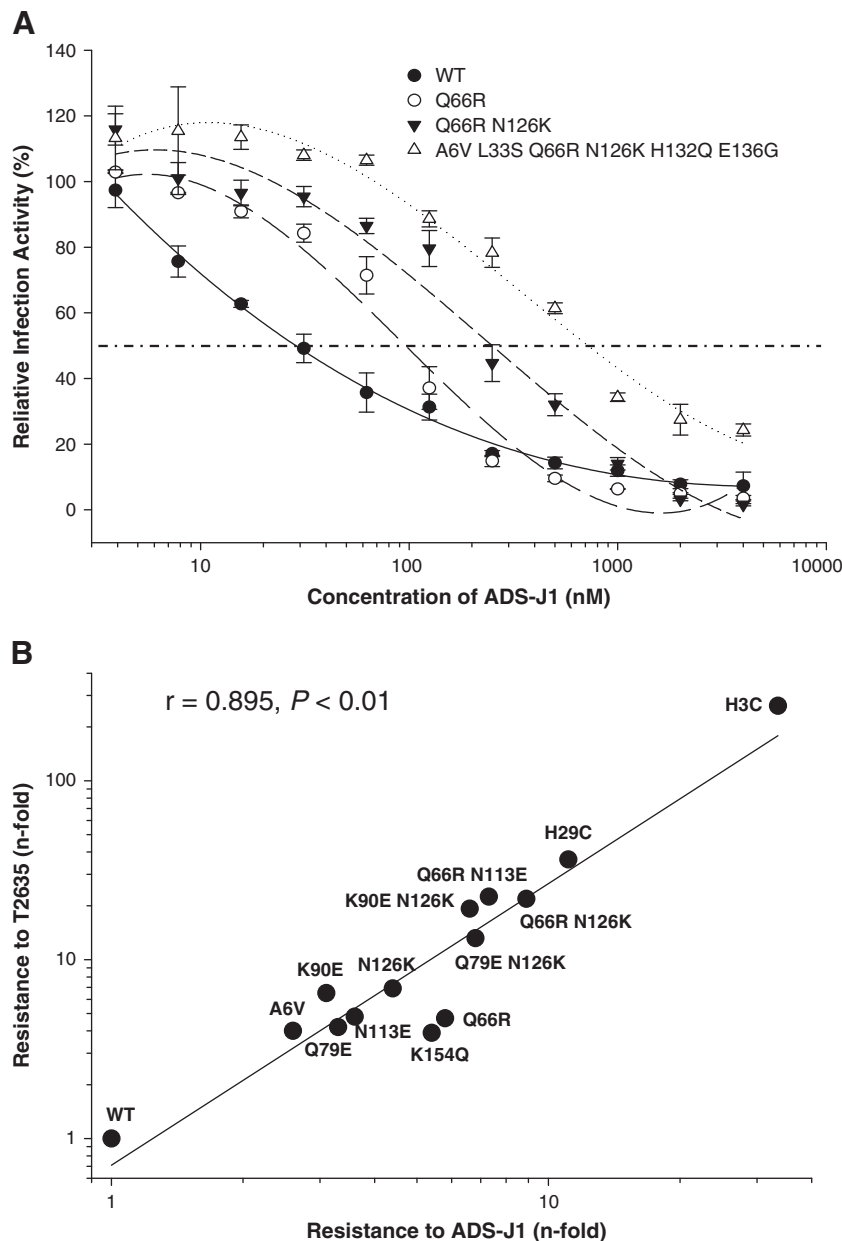


Fig. 2. Inhibitory activity of HIV-1 fusion inhibitor on infection of HIV-1 LAI clone and its mutants resistant to T2635. (A) Single-cycle infection experiments using TZM-b1 cells were performed to test the inhibitory activity of ADS-J1 on infection of the HIV-1 LAI clones with or without mutation. The relative infection activity of each virus clone in the presence of an inhibitory compound was calculated based on its luciferase activity (relative light units, RLU) in comparison with that of the virus in the absence of the inhibitory peptide. Error bars represent the standard deviation (SD) from at least three independent experiments. (B) Correlation between the viral resistance to T2635 and resistance to ADS-J. Pearson product-moment correlation coefficient (r) was calculated using Excel.

mutants, including N36(Q64A)Fd, N36(Q64L)Fd, N36(A67G)Fd, N36(A67S)Fd and N36(Q66R)Fd in the absence of ADS-J1, suggesting that these mutations do not significantly affect the ability of NHR-trimer to form 6-HB with CHR peptide. However, in the presence of increasing concentration of ADS-J1, the density of the upper bands (corresponding to the bands of 6-HBs) became weaker and weaker, while that of the lower bands (corresponding to the bands of unbound C34 peptide) became stronger and stronger. For the control N36Fd trimer and C34 at 100 μ M binding to the mutant N36Fd trimers and less potent in blocking (Fig. 4A), while for mutant peptide N36(A67G)Fd, ADS-J1 could fully inhibit 6-HB formation at 200 or 400 μ M (Fig. 4B–F). These results suggest that ADS-J1 is less effective in 6-HB

formation between the C34 peptide and the mutated N36Fd trimers than wild-type N36Fd trimer.

3.4. Decreased inhibitory activity of ADS-J1 in blocking interaction between C34 and N36Fd trimer mutants

To further identify the resistance mechanism of ADS-J1, we studied the properties of NHR-peptide N36Fd trimer and its mutants using CD spectroscopy. Our previous studies have demonstrated that ADS-J1 significantly interfered with the interaction between N36 and C34 when ADS-J1 was mixed with N36 before addition of C34 [38]. In the present study, we analyzed the secondary structures of the 6-HB formed by the interaction of C34 with N36Fd trimer, or its mutants. Our results demonstrated

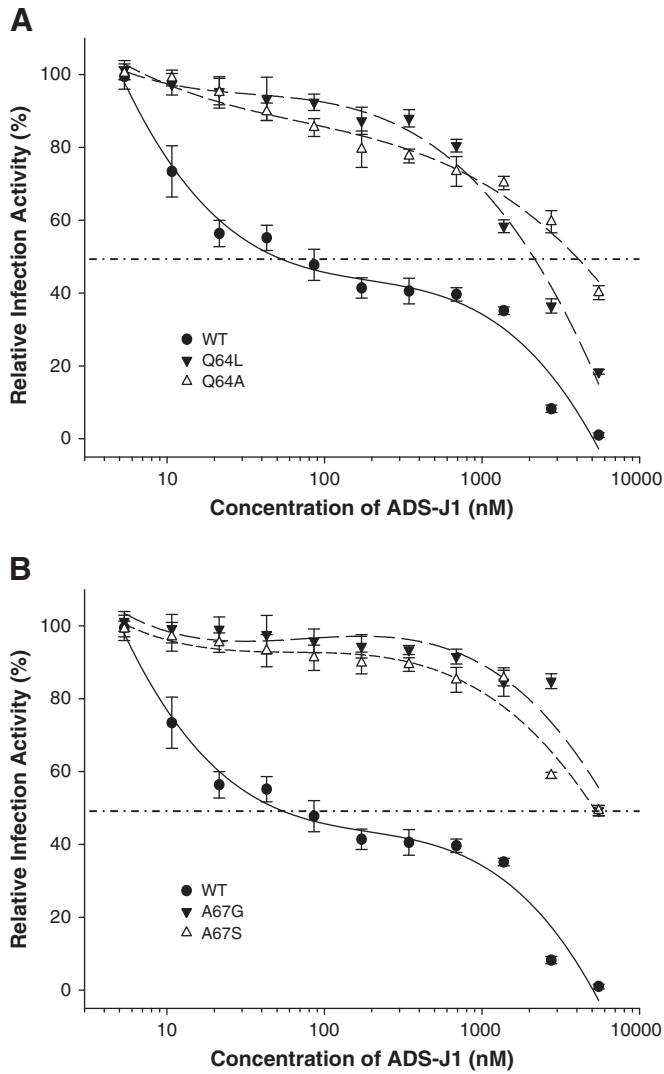


Fig. 3. Inhibitory activity of HIV-1 fusion inhibitor on infection of HIV-1 pseudoviruses expressing wild-type and mutated gp41. Single-cycle infection experiments using TZM-b1 cells were performed to test the inhibitory activity of ADS-J1 on infection of the viruses with mutation at the position of 64 (A) and 67 (B) in the gp41 pocket region. The relative infection activity of each virus in the presence of an inhibitory compound was calculated based on its relative light units (RLU) in comparison with that of the virus in the absence of the inhibitory peptide. Error bars represent the standard deviation (SD) from at least three independent experiments.

that mutations of the residues Q64, A67, and Q66 could affect the conformation and stability of the 6-HB formed by N36Fd trimer and C34, as indicated by the reduced α -helicity of the 6-HBs formed between C34 and the N36Fd trimers with mutations (Fig. 5, Table 3). ADS-J1 could interfere with the interaction between C34 and N36Fd trimer, as shown by the

Table 2

The resistance of the NL4-3 variants with mutations in the pocket-forming region of the HIV-1 gp41 NHR domain to the HIV-1 fusion inhibitors targeting gp41.

Virus	ADS-J1		C34		T20	
	IC ₅₀ (nM)	Resistance (n-fold)	IC ₅₀ (nM)	Resistance (n-fold)	IC ₅₀ (nM)	Resistance (n-fold)
WT	60 ± 0.01	1.0	10 ± 0.01	1.0	17 ± 0.01	1.0
Q64A	3602 ± 0.08	60.0	2100 ± 0.08	210.0	77 ± 0.03	4.5
Q64L	1773 ± 0.06	29.6	1470 ± 0.05	147.0	48 ± 0.02	2.8
A67G	5475 ± 0.09	91.3	2435 ± 0.09	243.5	58 ± 0.02	3.4
A67S	4639 ± 0.08	77.3	1025 ± 0.06	102.5	21 ± 0.01	1.2

IC₅₀ values are presented in nM. Results are derived from experiments performed in duplicate and are representative of at least three independent experiments.

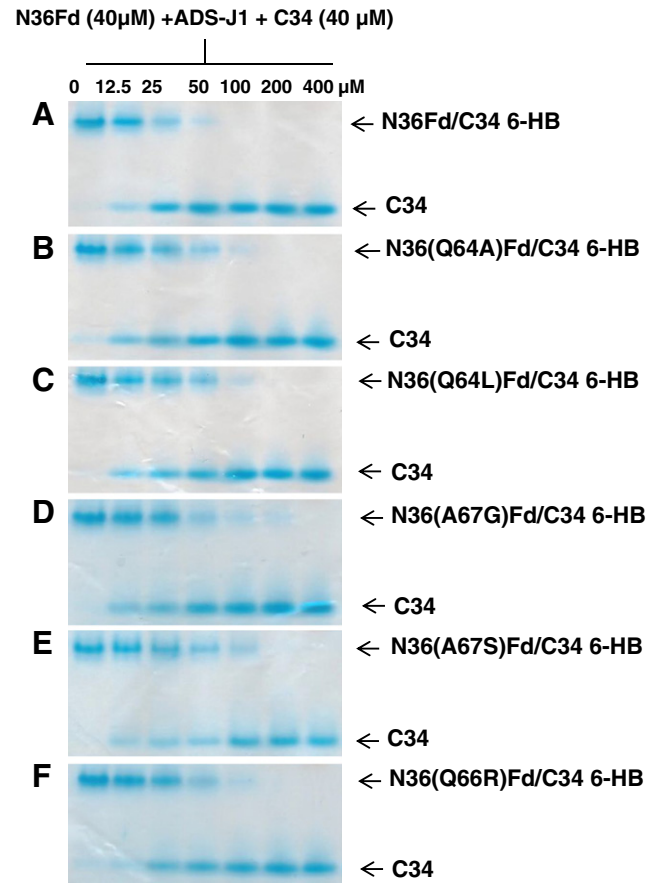


Fig. 4. Dose-dependent inhibition of 6-HB formation by ADS-J1, as assessed by N-PAGE. N36Fd trimer (A) or its mutants (B–F), and ADS-J1 with graded concentrations from 0 to 400 μ M were preincubated at 37 °C for 30 min before addition of C34. The mixture was then incubated at 37 °C for 30 min. The final concentration of the peptides was 40 μ M.

reduction of the α -helicity of the 6-HB formed between C34 and N36Fd trimer from 92.8% to 56.2% after the addition of ADS-J1. However, addition of ADS-J1 to the mixture of C34 and mutated N36Fd trimers caused no significant reduction of α -helicity (Table 3), suggesting that the binding of ADS-J1 to N36Fd trimer was reduced because of mutations in N36Fd trimer.

One may notice that N36Fd-trimer does not show a typical α -helical spectrum, possibly because of the interference of Fd's β -sheet spectrum [35] with the α -helical signals of N36-trimer. We previously showed that subtraction of the β -sheet spectra of free Fd peptide from that of N36Fd, a typical α -helical spectrum was revealed [8]. Our previous studies also demonstrated that N36Fd form stable trimers as determined by sedimentation velocity and SDS-PAGE analysis. Because N36Fd can form NHR-trimer, its anti-HIV-1 activity is about 15-fold higher than the free N36 peptide [8].

3.5. Reduced effectiveness of ADS-J1 in binding of N36Fd trimer with mutations in gp41 pocket region

We further detected the binding affinity of ADS-J1 to N36Fd trimer and its mutants by isothermal titration calorimetry. In comparison with the binding constant of wild-type N36Fd trimer ($5.23 \times 10^6 \text{ M}^{-1}$), the binding constant of ADS-J1 to the five mutants was reduced about 2–10-fold (5.68×10^5 – $1.92 \times 10^6 \text{ M}^{-1}$) (Fig. 6 and Table 4). The titration experiments revealed that the stoichiometry of the binding of ADS-J1 with N36Fd trimer and its mutants was 1.5–2.7:1, rather than 1:1, indicating that about two small molecules interact with one N36Fd trimer or its mutant.

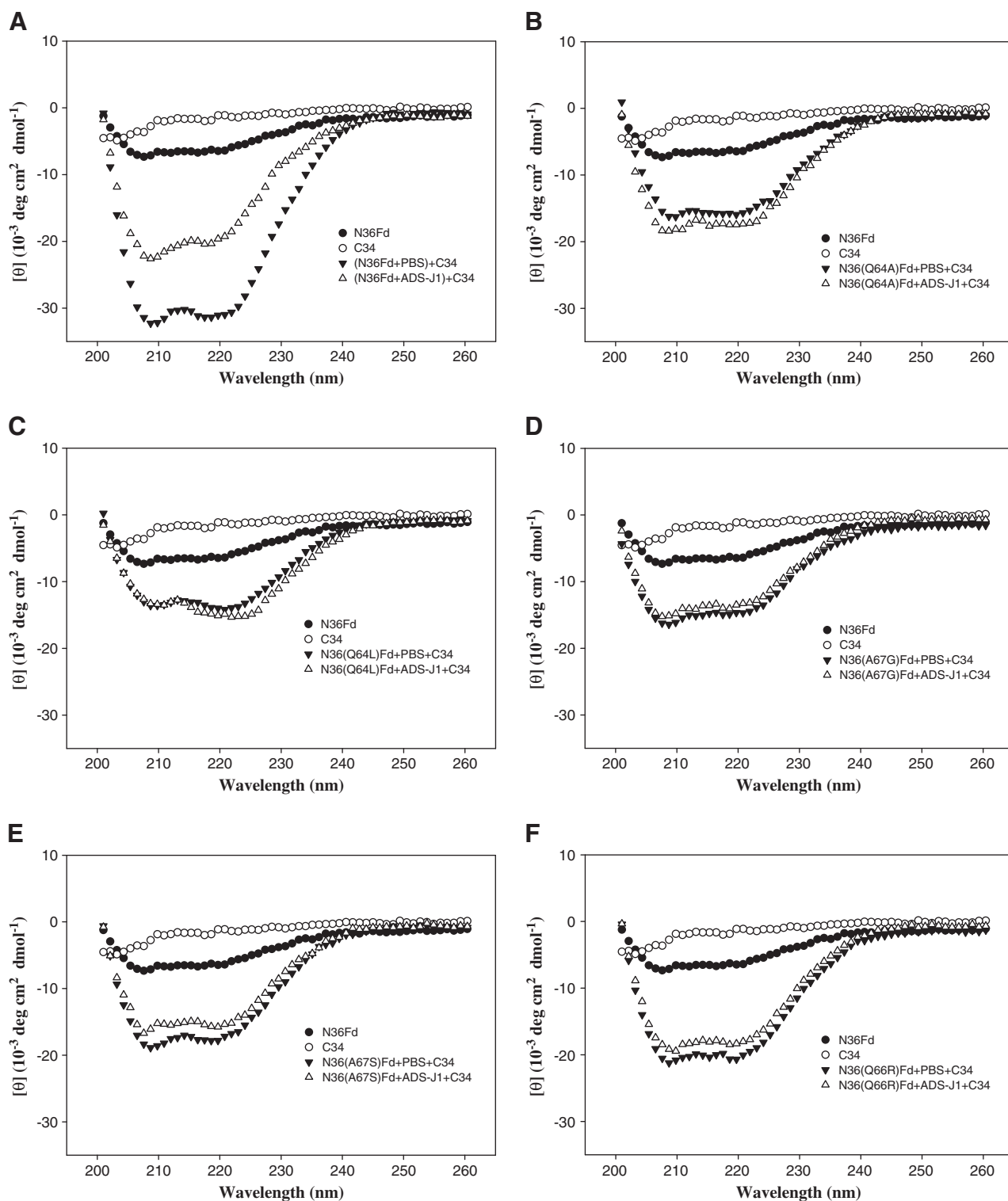


Fig. 5. ADS-J1 interfered with conformational change during the interaction between N36Fd trimer, N36Fd trimer mutants and C34, as detected by CD spectroscopy. (A) N36Fd trimer was incubated with ADS-J1 or PBS at 37 °C for 30 min before addition of C34, followed by additional incubation at 37 °C for 30 min. (B–F) The mutants of N36Fd trimer, including N36(Q64A) Fd, N36(Q64L)Fd, N36(Q66R)Fd, N36(A67G)Fd, N36(A67S)Fd, were incubated with ADS-J1 or PBS at 37 °C for 30 min before addition of C34, followed by additional incubation at 37 °C for 30 min. The final concentrations of each peptide (N- or C-peptide) and ADS-J1 were 10 μ M and 50 μ M, respectively. All of them were dissolved in PBS (pH 7.2). The α -helicity was calculated from the CD signal as described in [Materials and methods](#).

4. Discussion

HIV-1 entry into the target cell is initiated by binding of its Env protein surface subunit gp120 to the primary receptor CD4 and coreceptor,

CCR5 or CXCR4, triggering a series of confirmation changes of its Env protein transmembrane subunit gp41. Such changes involve insertion of the fusion peptide into the target cell membrane, and formation of the gp41 NHR-trimer as the transit fusion-intermediate state and the

Table 3
The α -helical content of N36Fd trimer and its mutants, as well as their mixtures with C34.

N-Peptide	$[\theta]_{222} \text{ nm} (\times 10^3)$	α -Helicity	Change of α -helicity
N36Fd + PBS + C34	-30.6	92.8%	
N36Fd + ADS-J1 + C34	-18.5	56.2%	36.6%
N36(Q64A)Fd + PBS + C34	-15.3	46.4%	
N36(Q64A)Fd + ADS-J1 + C34	-17.2	52.2%	-5.8%
N36(Q64L)Fd + PBS + C34	-15.3	46.4%	
N36(Q64L)Fd + ADS-J1 + C34	-13.9	42.2%	4.2%
N36(A67G)Fd + PBS + C34	-14.4	43.9%	
N36(A67G)Fd + ADS-J1 + C34	-13.2	40.0%	3.9%
N36(A67S)Fd + PBS + C34	-16.8	50.8%	
N36(A67S)Fd + ADS-J1 + C34	-15.2	46.1%	4.7%
N36(Q66R)Fd + PBS + C34	-19.5	59.1%	
N36(Q66R)Fd + ADS-J1 + C34	-17.7	53.7%	5.4%

All experiments were performed with 10 μM peptide solutions in phosphate buffered saline (pH 7.0).

6-HB between the gp41 NHR and CHR domains as the fusion core, bringing the viral and target cell membranes into close proximity for fusion [7,21]. A deep hydrophobic pocket ($\sim 16\text{-}\text{\AA}$ long, $\sim 7\text{-}\text{\AA}$ wide, and $5\text{-}6\text{-}\text{\AA}$ deep) in the groove on the gp41 NHR-trimer that is formed by a cluster of conserved hydrophobic residues [6,40] plays an important role in stabilization of the gp41 6-HB formation and gp41-mediated membrane fusion, as well as HIV-1 replication [5,17,34]. Therefore, this pocket has been recognized as an attractive target for developing HIV fusion inhibitors [5,20,25,26].

Using the gp41 pocket as the target in a computer-aided virtual screening method, together with an ELISA-based gp41 6-HB inhibition assay, we identified a small-molecule compound, ADS-J1, which inhibited HIV-Env-mediated cell-cell fusion and HIV-1 replication at low μM level [9,19]. The mechanism studies have shown that ADS-J1 binds to IQN17, a trimeric peptide containing the gp41 pocket region, by conjugating the GCN4 trimerization motif (IQ) with a 17-aa gp41 pocket-forming sequence (N17: aa 564–581) [11], and inhibited binding of PIE7, a short D peptide [11], with the gp41 pocket on IQN17 [38]. Binding of ADS-J1 to the pocket-containing NHR-peptide could block 6-HB formation between the NHR- and CHR-peptides [9]. Computer modeling analysis indicated that the negatively charged sulfonic acid group of ADS-J1 could interact with the positively charged side chain of K574 in the pocket region, but that the mutation of K574D resulted in the abrogation of ADS-J1 binding to the gp41 pocket region [38]. All of these results suggest that ADS-J1 mainly targeted the gp41 pocket region.

However, Este and colleagues argued that ADS-J1 targeted neither the pocket region nor any other regions in gp41. Instead, they hypothesized that it targets the V3 loop region of gp120 because it failed to induce resistant mutations in the gp41 pocket region, while it could induce resistant mutations in multiple regions of gp120, particularly the highly variable V3 loop region. These results were based on passage of an HIV-1 strain resistant to AR177, an anti-HIV-1 polyanionic oligonucleotide with primary target in gp120, in MT-4 cells for more than 8 months in the presence of ADS-J1 [3] or the related HIV-1 strains [14]. However, we are not convinced by their results because they did not use a peptide or small molecule compound that mainly targets the gp41 pocket as a control in their experiments. Nevertheless, HIV-1 escape does not necessarily take place at the drug binding site. For example, the CCR5 inhibitor (vicriviroc) can induce drug-resistant mutations in the fusion peptide (FP) of gp41 (e.g., M518V or F519L) [2], and some vicriviroc-resistant HIV-1 strains contain mutations in both FP and NHR domain of gp41 (e.g., M518V, F519L, and V535M) [1].

It has been well recognized that HIV fusion inhibitors targeting the gp41 pocket have a high barrier to resistance since it is extremely difficult to induce mutations in the gp41 pocket sequence, which is highly conserved. Moreover, mutation in this region, especially the residues of the N-helix that line the side chain and right wall of the pocket,

might result in the loss of viral function [34]. Indeed, the mutations induced by PBD-containing CHR peptides are not located in the pocket region of gp41, but rather in other regions in gp41, or even in some regions responsible for altering 6-HB formation and the time window of the fusion intermediate state [12,13]. Dwyer et al. obtained an HIV-1 strain with weak resistance (8.3-fold) to T2544, a PBD-containing CHR-peptide, after they passaged the viruses in cell cultures in the presence of T2544 for almost 8 months, while the related mutation sites could not be defined [10]. Similarly, Eggink et al. passaged T20- or T1249-resistant HIV-1 in SupT1 T-cell cultures in the presence of T2635, another PBD-containing CHR-peptide [10] at escalating concentrations for 6 months. Several T2635-resistant strains were obtained with multiple mutations in several gp41 regions, but only one of the mutations (Q66R) was located in the gp41 pocket region [12]. Interestingly, T2635 could also induce mutations in the V1 and V3 loops of gp120 (e.g., S148E/I/R and A316T), but cause no substitutions in the putative gp41-binding sites in gp120, including the C1 and C5 domains [16,22]. A mutation at the base of the V3 domain in gp120 could partially compensate for the loss of virus infectivity caused by T2635-resistant mutations in gp41 [12]. Therefore, the mutations in the V3 loop region of gp120 induced by ADS-J1, as observed by Armand-Ugon et al. [3], may also be a strategy used by HIV-1 to compensate for the loss of viral infectivity caused by ADS-J1-resistant mutations in gp41. Reeves et al. have observed that gp120/coreceptor affinity is correlated with sensitivity of coreceptor antagonist and HIV fusion inhibitor T20 since the enhanced affinity leads to more rapid fusion kinetics and shorter time window for fusion intermediate state, resulting in reduced sensitivity of the HIV-1 fusion inhibitors targeting the fusion intermediate. Viruses with mutations in V3 loop and the bridging sheet region of gp120 that result in gp120's binding affinity for CCR5 exhibited altered sensitivity to T20 [37].

In the present study, we first tested the sensitivity of these T2635-resistant HIV-1 strains to ADS-J1. Surprisingly, all the T2635-resistant HIV-1 strains tested were also resistant to ADS-J1 (Table 1, Fig. 2A), and their resistance to ADS-J1 and T2635 was well correlated ($r = 0.895$, $P < 0.01$, Fig. 2B). These results suggest that ADS-J1 and T2635 inhibit HIV-1 by a similar mechanism and may share the same target: the gp41 pocket region. We then constructed 4 HIV-1 pseudoviruses by mutating the residues at position 64 (Q64A or Q64L) or 67 (A67G or A67S) in the gp41 pocket region, both of which are located at the *c* and *f* positions, respectively, in the NHR helix wheel. Mutations at these sites are not expected to affect the interaction between NHR and CHR (Fig. 1B). We previously demonstrated that these HIV-1 mutants retained substantial infection and were highly resistant to the PBD-containing CHR peptides, such as CP32M, but sensitive to PBD-lacking CHR peptide, T20 [30,43]. Interestingly, all these mutants were highly resistant to ADS-J1 and C34, but relatively sensitive to T20 (Table 2 and Fig. 3). Like C34, these data indicate that ADS-J1 inhibits HIV-1 fusion by mainly targeting the gp41 pocket.

We subsequently constructed a gp41 NHR-trimer by conjugating the NHR-peptide N36 with a trimerization motif, foldon (Fd), designated N36Fd, and several mutant N36Fds, including N36(Q64A)Fd, N36(Q64L)Fd, N36(A67G)Fd, N36(A67S)Fd, and N36(Q66R)Fd. Similar to the wild-type N36Fd trimer, these NHR-trimers with mutations in the pocket region could also interact with the CHR-peptide C34 to form 6-HB. However, the inhibitory activity of ADS-J1 on 6-HB formation between the mutant NHR-trimer and C34 was lower than that on the 6-HB formation between the wild-type NHR-trimer and C34 as detected by N-PAGE analysis (Fig. 4). Similarly, the ability of ADS-J1 to interfere with the interaction between wild-type N36Fd trimer and C34 was lower than its ability to interfere with the interaction between the mutant N36Fd trimer and C34 as determined by CD spectroscopy (Table 3 and Fig. 5). Furthermore, the binding affinity of the mutant N36Fd trimer to ADS-J1 was lower than that of the wild-type N36Fd trimer as analyzed by isothermal titration calorimetry (Table 4 and Fig. 6). All of these findings suggest that the gp41 pocket is the main target of

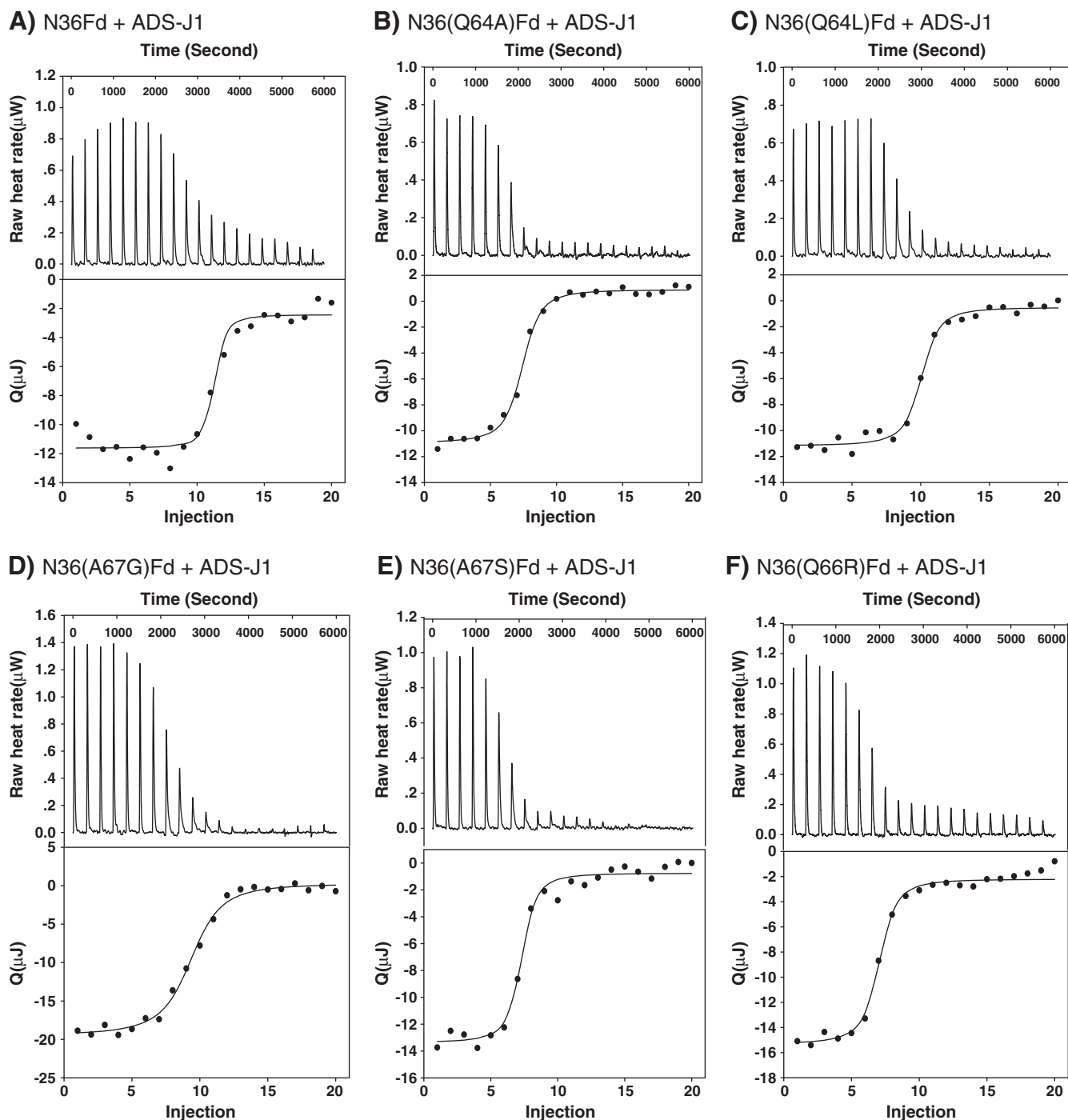


Fig. 6. The interaction of ADS-J1 with N36Fd trimer and its mutants. The binding of ADS-J1 to N36Fd trimer (A) and its mutants (B–F) was measured by isothermal titration calorimetry assay (ITC). Upper panel shows the titration traces when ADS-J1 dissolved in PBS was injected into the N36 solution. Lower panel shows the binding affinity of ADS-J1 to the N36Fd trimer and its mutants.

ADS-J1 and that mutations in the pocket region result in the decreased binding of ADS-J1 to this pocket.

In conclusion, ADS-J1, a small-molecule HIV-1 fusion inhibitor, inhibits HIV-1 infection by binding to the conserved hydrophobic pocket in the gp41 NHR-trimer at the fusion-intermediate state and by blocking 6-HB fusion core formation between the viral gp41 NHR and CHR domains. Mutations in the pocket region of gp41 may affect the pocket-binding activity and HIV-1 fusion inhibitory activity of ADS-J1. Therefore, this study provides important information for elucidation of the mechanism of ADS-J1 and suggests that ADS-J1 may serve as a

lead compound for designing small molecule HIV fusion inhibitors targeting the gp41 pocket and as a molecule probe for investigating the fusogenic mechanisms of HIV-1.

Acknowledgements

This work was supported by grants from the Natural Science Foundation of China (81173098, 81261120382, 81373456) and the National 973 Program of China (2012CB519001).

Table 4
Thermodynamic parameters of ADS-J1 binding to N36Fd trimer and its mutants.

Peptide	Molar ratio	K(M)	ΔG	ΔH (KJ/mol)	ΔS (J/mol)
N36Fd + ADS-J1	2.610	1.91×10^{-7}	-4653.53	-9.20	97.77
N36(Q64A)Fd + ADS-J1	1.639	7.81×10^{-7}	-4230.89	-11.84	77.23
N36(Q64L)Fd + ADS-J1	2.299	5.21×10^{-7}	-4352.12	-10.66	84.53
N36(Q66R)Fd + ADS-J1	1.550	7.19×10^{-7}	-4254.06	-13.13	73.53
N36(A67S)Fd + ADS-J1	1.666	8.70×10^{-7}	-4197.80	-12.96	72.55
N36(A67G)Fd + ADS-J1	2.150	1.76×10^{-6}	-3985.73	-19.78	43.84

References

- [1] C.G. Anastassopoulou, T.J. Ketas, R.S. Depetris, A.M. Thomas, P.J. Klasse, J.P. Moore, Resistance of a human immunodeficiency virus type 1 isolate to a small molecule CCR5 inhibitor can involve sequence changes in both gp120 and gp41, *Virology* 413 (2011) 47–59.
- [2] C.G. Anastassopoulou, T.J. Ketas, P.J. Klasse, J.P. Moore, Resistance to CCR5 inhibitors caused by sequence changes in the fusion peptide of HIV-1 gp41, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 5318–5323.
- [3] M. Armand-Ugon, I. Clotet-Codina, C. Tintori, F. Manetti, B. Clotet, M. Botta, J.A. Este, The anti-HIV activity of ADS-J1 targets the HIV-1 gp120, *Virology* 343 (2005) 141–149.
- [4] C.E. Baldwin, R.W. Sanders, Y. Deng, S. Jurriaans, J.M. Lange, M. Lu, B. Berkhout, Emergence of a drug-dependent human immunodeficiency virus type 1 variant during therapy with the T20 fusion inhibitor, *J. Virol.* 78 (2004) 12428–12437.
- [5] D.C. Chan, C.T. Chutkowski, P.S. Kim, Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 15613–15617.
- [6] D.C. Chan, D. Fass, J.M. Berger, P.S. Kim, Core structure of gp41 from the HIV envelope glycoprotein, *Cell* 89 (1997) 263–273.
- [7] D.C. Chan, P.S. Kim, HIV entry and its inhibition, *Cell* 93 (1998) 681–684.
- [8] X. Chen, L. Lu, Z. Qi, H. Lu, J. Wang, X. Yu, Y. Chen, S. Jiang, Novel recombinant engineered gp41 N-terminal heptad repeat trimers and their potential as anti-HIV-1 therapeutics or microbicides, *J. Biol. Chem.* 285 (2010) 25506–25515.
- [9] A.K. Debnath, L. Radigan, S. Jiang, Structure-based identification of small molecule antiviral compounds targeted to the gp41 core structure of the human immunodeficiency virus type 1, *J. Med. Chem.* 42 (1999) 3203–3209.
- [10] J.J. Dwyer, K.L. Wilson, D.K. Davison, S.A. Freely, J.E. Seedorff, S.A. Wring, N.A. Tvermoes, T.J. Matthews, M.L. Greenberg, M.K. Delmedico, Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 12772–12777.
- [11] D.M. Eckert, V.N. Malashkevich, L.H. Hong, P.A. Carr, P.S. Kim, Inhibiting HIV-1 entry: discovery of D-peptide inhibitors that target the gp41 coiled-coil pocket, *Cell* 99 (1999) 103–115.
- [12] D. Eggink, I. Bontjer, J.P. Langedijk, B. Berkhout, R.W. Sanders, Resistance of human immunodeficiency virus type 1 to a third-generation fusion inhibitor requires multiple mutations in gp41 and is accompanied by a dramatic loss of gp41 function, *J. Virol.* 85 (2011) 10785–10797.
- [13] D. Eggink, J.P. Langedijk, A.M. Bonvin, Y. Deng, M. Lu, B. Berkhout, R.W. Sanders, Detailed mechanistic insights into HIV-1 sensitivity to three generations of fusion inhibitors, *J. Biol. Chem.* 284 (2009) 26941–26950.
- [14] E. Gonzalez-Ortega, M.P. Mena, M. Permany, E. Ballana, B. Clotet, J.A. Este, ADS-J1 inhibits HIV-1 entry by interacting with gp120 and does not block fusion-active gp41 core formation, *Antimicrob. Agents Chemother.* 54 (2010) 4487–4492.
- [15] Y. He, J. Cheng, H. Lu, J. Li, J. Hu, Z. Qi, Z. Liu, S. Jiang, Q. Dai, Potent HIV fusion inhibitors against Enfuvirtide-resistant HIV-1 strains, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 16332–16337.
- [16] E. Helseth, U. Olshevsky, C. Furman, J. Sodroski, Human immunodeficiency virus type 1 gp120 envelope glycoprotein regions important for association with the gp41 transmembrane glycoprotein, *J. Virol.* 65 (1991) 2119–2123.
- [17] H. Ji, W. Shu, T. Burling, S. Jiang, M. Lu, Inhibition of HIV-1 infectivity by the gp41 core: role of a conserved hydrophobic cavity in membrane fusion, *J. Virol.* 73 (1999) 8578–8586.
- [18] S. Jiang, K. Lin, N. Strick, A.R. Neurath, HIV-1 inhibition by a peptide, *Nature* 365 (1993) 113.
- [19] S. Jiang, K. Lin, L. Zhang, A.K. Debnath, A screening assay for antiviral compounds targeted to the HIV-1 gp41 core structure using a conformation-specific monoclonal antibody, *J. Virol. Methods* 80 (1999) 85–96.
- [20] S. Jiang, P. Siddiqui, S. Liu, Blocking viral entry: a complementary strategy for HIV therapy, *Drug Discovery Today* 1 (2004) 497–503.
- [21] S. Jiang, Q. Zhao, A.K. Debnath, Peptide and non-peptide HIV fusion inhibitors, *Curr. Pharm. Des.* 8 (2002) 563–580.
- [22] J.P. Julien, A. Cupo, D. Sok, R.L. Stanfield, D. Lyumkis, M.C. Deller, P.J. Klasse, D.R. Burton, R.W. Sanders, J.P. Moore, A.B. Ward, I.A. Wilson, Crystal structure of a soluble cleaved HIV-1 envelope trimer, *Science* 342 (2013) 1477–1483.
- [23] J.M. Kilby, S. Hopkins, T.M. Venetta, B. DiMassimo, G.A. Cloud, J.Y. Lee, L. Allredge, E. Hunter, D. Lambert, D. Bolognesi, T. Matthews, M.R. Johnson, M.A. Nowak, G.M. Shaw, M.S. Saag, Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry, *Nat. Med.* 4 (1998) 1302–1307.
- [24] S. Liu, L. Boyer-Chatenet, H. Lu, S. Jiang, Rapid and automated fluorescence-linked immunosorbent assay for high throughput screening of HIV-1 fusion inhibitors targeting gp41, *J. Biomol. Screen.* 8 (2003) 685–693.
- [25] S. Liu, S. Jiang, High throughput screening and characterization of HIV-1 entry inhibitors targeting gp41: theories and techniques, *Curr. Pharm. Des.* 10 (2004) 1827–1843.
- [26] S. Liu, W. Jing, B. Cheung, H. Lu, J. Sun, X. Yan, J. Niu, J. Farmer, S. Wu, S. Jiang, HIV gp41 C-terminal heptad repeat contains multifunctional domains: relation to mechanisms of action of anti-HIV peptides, *J. Biol. Chem.* 282 (2007) 9612–9620.
- [27] S. Liu, H. Lu, Y. Xu, S. Wu, S. Jiang, Different from the HIV fusion inhibitor C34, the anti-HIV drug Fuzeon (T-20) inhibits HIV-1 entry by targeting multiple sites in gp41 and gp120, *J. Biol. Chem.* 280 (2005) 11259–11273.
- [28] S. Liu, S. Wu, S. Jiang, HIV entry inhibitors targeting gp41: from polypeptides to small-molecule compounds, *Curr. Pharm. Des.* 13 (2007) 143–162.
- [29] S. Liu, Q. Zhao, S. Jiang, Determination of the HIV-1 gp41 postfusion conformation modeled by synthetic peptides: applicable for identification of the HIV-1 fusion inhibitors, *Peptide* 24 (2003) 1303–1313.
- [30] L. Lu, P. Tong, X. Yu, C. Pan, P. Zou, Y.H. Chen, S. Jiang, HIV-1 variants with a single-point mutation in the gp41 pocket region exhibiting different susceptibility to HIV fusion inhibitors with pocket- or membrane-binding domain, *Biochim. Biophys. Acta* 1818 (2012) 2950–2957.
- [31] M. Lu, S.C. Blacklow, P.S. Kim, A trimeric structural domain of the HIV-1 transmembrane glycoprotein, *Nat. Struct. Biol.* 2 (1995) 1075–1082.
- [32] M. Lu, P.S. Kim, A trimeric structural subdomain of the HIV-1 transmembrane glycoprotein, *J. Biomol. Struct. Dyn.* 15 (1997) 465–471.
- [33] M. Mink, S.M. Mosier, S. Janumpalli, D. Davison, L. Jin, T. Melby, P. Sista, J. Erickson, D. Lambert, S.A. Stanfield-Oakley, M. Salgo, N. Cammack, T. Matthews, M.L. Greenberg, Impact of human immunodeficiency virus type 1 gp41 amino acid substitutions selected during enfuvirtide treatment on gp41 binding and antiviral potency of enfuvirtide in vitro, *J. Virol.* 79 (2005) 12447–12454.
- [34] H. Mo, A.K. Konstantinidis, K.D. Stewart, T. Dekhtyar, T. Ng, K. Swift, E.D. Matayoshi, W. Kati, W. Kohlbrenner, A. Molla, Conserved residues in the coiled-coil pocket of human immunodeficiency virus type 1 gp41 are essential for viral replication and interhelical interaction, *Virology* 329 (2004) 319–327.
- [35] K. Papanikolopoulou, V. Forge, P. Goeltz, A. Mitraki, Formation of highly stable chimeric trimers by fusion of an adenovirus fiber shaft fragment with the foldon domain of bacteriophage T4 fibrin, *J. Biol. Chem.* 279 (2004) 8991–8998.
- [36] N. Ray, L.A. Blackburn, R.W. Doms, HR-2 mutations in human immunodeficiency virus type 1 gp41 restore fusion kinetics delayed by HR-1 mutations that cause clinical resistance to enfuvirtide, *J. Virol.* 83 (2009) 2989–2995.
- [37] J.D. Reeves, S.A. Gallo, N. Ahmad, J.L. Miamidian, P.E. Harvey, M. Sharron, S. Pohlmann, J.N. Sfakianos, C.A. Derdeyn, R. Blumenthal, E. Hunter, R.W. Doms, Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 16249–16252.
- [38] H. Wang, Z. Qi, A. Guo, Q. Mao, H. Lu, X. An, C. Xia, X. Li, A.K. Debnath, S. Wu, S. Liu, S. Jiang, ADS-J1 inhibits HIV-1 entry by interacting with the gp41 pocket region and blocking the fusion-active gp41 core formation, *Antimicrob. Agents Chemother.* 53 (2009) 4987–4998.
- [39] X. Wei, J.M. Decker, H. Liu, Z. Zhang, R.B. Arani, J.M. Kilby, M.S. Saag, X. Wu, G.M. Shaw, J.C. Kappes, Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy, *Antimicrob. Agents Chemother.* 46 (2002) 1896–1905.
- [40] W. Weissenhorn, A. Dessen, S.C. Harrison, J.J. Skehel, D.C. Wiley, Atomic structure of the ectodomain from HIV-1 gp41, *Nature* 387 (1997) 426–428.
- [41] C.T. Wild, D.C. Shugars, T.K. Greenwell, C.B. McDaniel, T.J. Matthews, Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection, *Proc. Natl. Acad. Sci. U. S. A.* 91 (1994) 9770–9774.
- [42] L. Xu, A. Pozniak, A. Wildfire, S.A. Stanfield-Oakley, S.M. Mosier, D. Ratcliffe, J. Workman, A. Joall, R. Myers, E. Smit, P.A. Cane, M.L. Greenberg, D. Pillay, Emergence and evolution of enfuvirtide resistance following long-term therapy involves heptad repeat 2 mutations within gp41, *Antimicrob. Agents Chemother.* 49 (2005) 1113–1119.
- [43] X. Yu, L. Lu, L. Cai, P. Tong, S. Tan, P. Zou, F. Meng, Y.H. Chen, S. Jiang, Mutations of Gln64 in the HIV-1 gp41 N-terminal heptad repeat render viruses resistant to peptide HIV fusion inhibitors targeting gp41 pocket, *J. Virol.* 86 (2012) 589–593.