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# Design and evaluation of antiretroviral peptides corresponding to the C-terminal heptad repeat region (C-HR) of human immunodeficiency virus type 1 envelope glycoprotein gp41

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ABSTRACT

Two α-helical heptad repeats, N-HR and C-HR, located in the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp41, play an important role in membrane fusion by forming a 6-helix bundle. C34, a peptide mimicking C-HR, inhibits the formation of the 6-helix bundle; thus, it has potential as a novel antiretroviral compound. In order to improve the inhibitory effect of C34 on HIV-1 replication, we designed new C34-derived peptides based on computational analysis of the stable conformation of the 6-helix bundle. Newly designed peptides showed a stronger inhibitory effect on the replication of recombinant viruses containing CRF01_AE, subtype B or subtype C Env than C34 or a fusion inhibitor, T-20. In addition, these peptides inhibited the replication of a T-20-resistant virus. We propose that these peptides could be applied to develop novel antiretroviral compounds to inhibit the replication of various subtypes of HIV-1 as well as of T-20-resistant variants.



## Introduction

The entry of human immunodeficiency virus type 1 (HIV-1) into cells is mediated by a viral envelope glycoprotein (Env) complex, consisting of a trimer of two non-covalently associated subunits, the gp120 surface glycoprotein and the gp41 transmembrane glycoprotein (Wyatt and Sodroski, 1998). After the binding of gp120 to the CD4 molecule and subsequently to a chemokine receptor, CCR5 or CXCR4, on the cell surface, the gp41 trimer forms an extended conformation of the three helices that allows a hydrophobic fusion peptide to be inserted into the target cell membrane, generating a pre-hairpin intermediate that is anchored to both cellular and viral membranes. After this step, gp41 is believed to start refolding to a more stable 6-helix bundle composed of the α-helical trimer of the N-terminal heptad repeat (N-HR) folded into an anti-parallel conformation with the three C-terminal heptad repeats (C-HR). This refolding brings the viral and cellular membranes into close proximity, leading to the formation of a fusion pore and resulting in the completion of the fusion process (Chan and Kim, 1998; Weiss, 2003).

The C-HR-derived 36-mer peptide, T-20 (DP-178, enfuvirtide) (Wild et al., 1993, 1994) and the 34-mer peptide, C34 (Chan et al., 1998; Malashkevich et al., 1998), suppress HIV-1 replication by inhibiting the process of membrane fusion. T-20 inhibits the formation of the 6-helix bundle as well as of the fusion pore, while C34 inhibits the formation of the 6-helix bundle (Kliger et al., 2001). T-20 is approved for clinical use as a HIV-1 fusion inhibitor, and is able to suppress the replication of HIV-1 variants with multi-drug resistance to reverse transcriptase and protease inhibitors (Kilby et al., 1998; Lalezari et al., 2003; Lazzarin et al., 2003); however, long-term therapy with T-20 resulted in the emergence of T-20-resistant HIV-1 variants (Greenberg and Cammack, 2004; Wei et al., 2002). C34 has not been approved for clinical use, but has the potential to be a novel HIV-1 fusion inhibitor. In order to improve the inhibitory effect of C34 on HIV-1 replication, we designed new C34-derived peptides by computational analysis.

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HIV-1 is characterized by extensive genetic heterogeneity (Gaschen et al., 2002), and is divided into four groups, M, N, O and P. The viruses in group M are further classified into many subtypes and circulating recombinant forms (CRFs). Among them, subtypes A, B, C and D, as well as CRF01_AE and CRF02_AG, are the major subtypes and CRFs, which are responsible for the worldwide HIV-1 pandemic (Hemelaar et al., 2006; McCutchan, 2006). Env is the most variable HIV-1 protein with typical intersubtype and intrasubtype differences reaching 35% and 20%, respectively (Gaschen et al., 2002). In this report, we tested the inhibitory effect of newly designed C34-derived peptides for *in vitro* replication of the following viruses: CRF01_AE viruses, which are prevalent in southeast Asian countries, including Thailand; subtype B viruses, which are prevalent in the Americas, Northern Europe and Australia, and are the most extensively studied viral population; and subtype C viruses, which are prevalent in Africa and India, and are the most prevalent viral population in the world; as well as a T-20-resistant variant of the subtype B reference strain, pNL4-3 (Adachi et al., 1986).

## Results and discussion

### *Screening of newly designed C34-derived peptides*

We designed ten C34-derived peptides, which were expected to interact with N-HR more strongly than the original C34 peptide (Table 1), based on the results of molecular dynamics analysis of the stable conformation of the 6-helix bundle consisting of N-HR and C-HR (Kawashita, N. et al., submitted). Namely, stronger interaction energies were expected for the association of N-HR and these peptides compared to the association of N-HR and C34. First, we examined the inhibitory effect of these peptides on the replication of CRF01_AE Env (AE-Env)-recombinant, luciferase reporter viruses. As controls, a fusion inhibitor, T-20, and the original C34 were used. The results of neutralization tests showed that three newly designed peptides, KYK01, KYK02 and KYK03, inhibited the replication of AE-Env-recombinant viruses efficiently (Fig. 1A), while the seven remaining peptides did not show a stronger inhibitory effect on viral replication than the original C34 (data now shown). KYK01 inhibited the replication of six AE-Env-recombinant viruses more efficiently than T-20 (Fig. 1A). In addition, KYK02 and KYK03 inhibited viral replication more efficiently than the original C34 (Fig. 1A).

We next examined the inhibitory effect of three newly designed peptides on the replication of subtype B Env (B-Env)- and subtype C Env (C-Env)-recombinant viruses. The results showed that KYK01, KYK02 and KYK03 inhibited the replication of most B-Env-recombinant viruses more efficiently than the original C34 (Fig. 1B). In addition, these peptides showed a similar inhibitory effect to T-20 on the replication of most B-Env-recombinant viruses (Fig. 1B). Furthermore, these peptides inhibited the replication of most C-Env-recombinant viruses more efficiently than the original C34 or T-20 (Fig. 1C). Taking these results together, three newly designed peptides, KYK01, KYK02 and KYK03, inhibited AE-Env-, B-Env- and C-Env-recombinant viruses more efficiently than the original C34 (Fig. 1). In addition, these peptides showed a similar or higher inhibitory effect on most of the recombinant viruses compared to T-20 (Fig. 1). The interaction energies for the association of the following pairs of molecules, KYK01/N-HR, KYK02/N-HR, KYK03/N-HR and C34/N-HR, were expected to be −960 kcal/mol, −1388 kcal/mol, −913 kcal/mol and −877 kcal/mol, respectively; therefore, the results of *in vitro* and *in silico* experiments were partially correlated.

**Table 1**
Amino acid sequence of C34-derived peptides used in the first screening test.

| | 117 150[a] |
|---|---|
| C34 | WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL |
| KYK01 | ....EK..D...N..YN.L.....E.....[b] |
| KYK02 | RI..E...DR..KE..R.L....R..DR..KD.K |
| KYK03 | ..Q.E...E...DE.YR...Q..... |
| KYK04 | RI..E.....K..YE.LT.....R..KD.. |
| KYK05 | RI..E...S...NQ.YEILT.....DR..KD.. |
| KYK06 | R...E...D.....YN..... |
| KYK07 | ....E.....GETYR...K.....R... |
| KYK08 | RQQQE..LER..RRTYREL.RE.Q.R.E..R... |
| KYK09 | RQQQE..LERR.RRTRREL.RE.Q.R.E..R... |
| KYK10 | RQRRE..TRRR.RRTRRRT.RERR...R..R... |

[a]Amino acid residues were numbered according to the pNL4-3 gp41 sequence.
[b]Amino acid sequence of C34-derived peptides used in this study was aligned with that of C34. Dots denote amino acid identity.

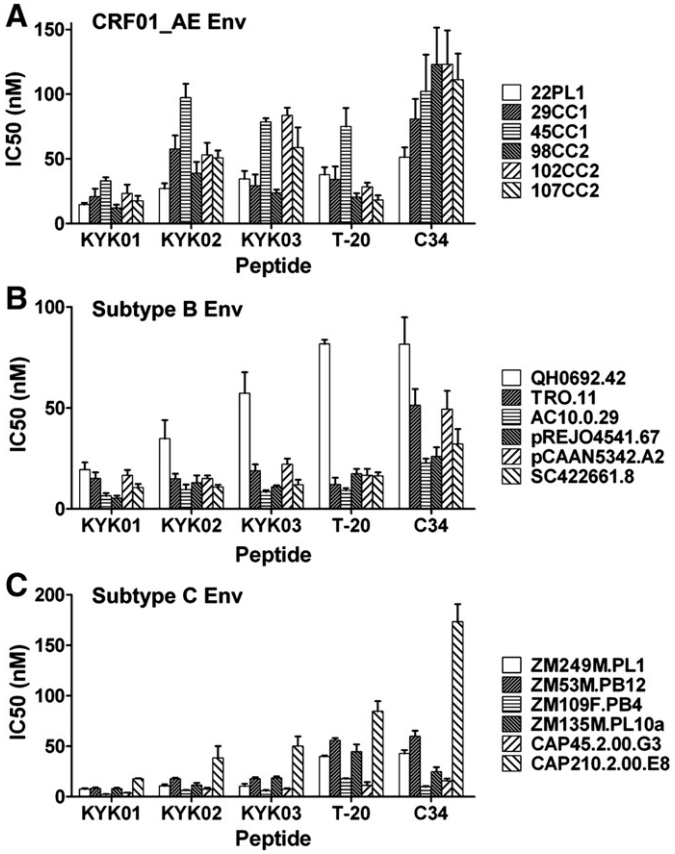


**Fig. 1.** Three newly designed C34-derived peptides inhibit the replication of AE-Env-, B-Env- and C-Env-recombinant viruses more efficiently than the original C34. The inhibitory effect of the indicated peptides on the replication of six AE-Env- (A), six B-Env- (B) and six C-Env-recombinant viruses (C) was evaluated, and the IC50 values of the peptides for inhibiting viral replication were calculated, as described in Materials and methods. Data are presented as the means and standard errors (error bars) of three independent experiments.

The extent of the inhibitory effect of C34-derived peptides as well as of T-20 varied among the recombinant viruses, suggesting that the N-HR of the HIV-1 Env studied may show variations in the amino acid sequence. To clarify this possibility, we compared the N-HR, as well as C-HR, sequence of the HIV-1 Env studied. The results showed that high levels of amino acid variations were observed in C-HR, while N-HR sequences were relatively conserved among the HIV-1 Env studied (Table 2). We examined the correlation between variations in the N-HR sequences and the neutralization susceptibility of the virus to the peptides; however, no correlation was observed.

### *KYK01, KYK02 and KYK03 fail to inhibit the replication of the pNL4-3-derived, T-20-resistant virus*

We next examined the inhibitory effect of newly designed C34-derived peptides on the replication of a T-20-resistant virus. To this end, we constructed the T-20-resistant virus, pNL-T20R, by

**Table 2**
N-HR and C-HR sequences of HIV-1 Env studied.

| | | |
|---|---|---|
| N-HR | | |
| | | 29 82[a] |
| B[b] | pNL4-3 | QARQLLSDIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQQLL |
| | pNL-T20R | .....E...S.....[c] |
| AE | 22PL1 | .....G.....S.....M.....V.....R.. |
| | 29CC1 | .....G.....S.....V.....Q..KF. |
| | 45CC1 | .....G.....S.....V.....TF. |
| | 98CC2 | .....G.....S.....V.....Q..KF. |
| | 102CC2 | .....G.....S.....V.....KF. |
| | 107CC2 | .....G.....S.....M.....L.....KF. |
| B | QH0692.42 | .....G.....M.....V.....R..... |
| | TRO.11 | ...L...G.....M.....V.....R..... |
| | AC10.0.29 | ...L...G.....V.....R..... |
| | pREJO4541.67 | .....G.....S.....V..M.S..... |
| | pCAAN5342.A2 | ...L...G.....V..I..... |
| | SC422661.8 | ...L...G.....V.....R..... |
| C | ZM249M.PL1 | .....G.....M.....V..I..... |
| | ZM53M.PB12 | .....G.....M.....V..I..... |
| | ZM109F.PB4 | .....G.....S.....V..I....Q..... |
| | ZM135M.PL10a | ....V..G.....S.....M.....V..I..... |
| | CAP45.2.00.G3 | .....G.....S.....M.....T.V..I..... |
| | CAP210.2.00.E8 | .....G.....S.....M.....T.V..I..... |
| C-HR | | |
| | | 117 162 |
| B | pNL4-3 | WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF |
| | pNL-T20R | ..... |
| AE | 22PL1 | .T..E...S...NQ.YNILT.....D...K..M..... |
| | 29CC1 | .V..E...S...N..YGILA...T...R...S..A..H..... |
| | 45CC1 | .IQ.E...S...NQ.YD..T.....D...KD..... |
| | 98CC2 | .R..EK..S...DK.YEILT.....D...KD..A..Q..... |
| | 102CC2 | .A..E...S...NT.YEILT...I..DS..KK..... |
| | 107CC2 | .T..E...S...NQ.YDILT.....D...K.....D.. |
| B | QH0692.42 | ..Q..K.....N..Y..L.D.....H..... |
| | TRO.11 | ....E...D...D..YI.L.K..I.....S..... |
| | AC10.0.29 | ..Q.....S.....YT.....A.....N..... |
| | pREJO4541.67 | .R..EK..D...D..YT...K..... |
| | pCAAN5342.A2 | ....EK..D...GI.YN.L.....D...K.....T.. |
| | SC422661.8 | .....YN.L.DA..... |
| C | ZM249M.PL1 | ..Q..K..S...QT.YN.L....S.....KD.....S.NN..... |
| | ZM53M.PB12 | ..Q..K.VS...KT.YK.L.K.....E..KD..A..S.NN..... |
| | ZM109F.PB4 | ..Q..K.VS...FT.YQ.L....Y...Q..K...A.N..ND..S.. |
| | ZM135M.PL10a | ..Q..K..S...DT.YK.LT...S..D...KD..A..S.KN..... |
| | CAP45.2.00.G3 | .IQ.....S..SNT.YK.L.G.....Q..KD..A..S.NN..... |
| | CAP210.2.00.E8 | ..Q.....NT.YR.L.D.....D..A....Q...S.. |

[a]Amino acid residues were numbered according to the pNL4-3 gp41 sequence.
[b]Subtype classification of HIV-1 Env studied. AE, B and C denote CRF01_AE, subtype B, and subtype C, respectively.
[c]N-HR and C-HR sequences of HIV-1 Env studied were aligned with that of a subtype B reference strain, pNL4-3. Dots denote amino acid identity.

introducing two amino acid substitutions, V38E [amino acid substitution from valine (V) to glutamic acid (E) at position 38 of gp41] and N42S, which markedly reduced viral susceptibility to T-20 (Greenberg and Cammack, 2004), into pNL4-3. Neutralization tests revealed that pNL-T20R could efficiently replicate in the presence of 5 μM of T-20, original C34 or a newly designed peptide (data not shown). Namely, KYK01, KYK02 and KYK03 did not show an inhibitory effect on the replication of the T-20-resistant virus, although these peptides could efficiently inhibit the replication of most AE-Env-, B-Env- and C-Env-recombinant viruses tested (Fig. 1).

*Design of KYK01- and KYK02-derived peptides which inhibit the replication of the T-20-resistant virus*

We next attempted to modify KYK01 and KYK02, which efficiently inhibited most of the Env-recombinant viruses tested, to generate a peptide capable of inhibiting the replication of the T-20-resistant virus, pNL-T20R. Although KYK01 showed the strongest inhibitory effect on all Env-recombinant viruses among the peptides tested (Fig. 1), its solubility was quite low (data not shown). In order to improve the solubility of KYK01, we designed two peptides, KYK01-1 and KYK01-2 (Table 3). KYK01-1 contains additional amino acid residues in its C-terminus, while KYK01-2 contains a few hydrophilic residues in its C- and N-terminal regions (Table 3); however, the solubility of KYK01-1 was rather deteriorated, while KYK01-2 did not show a strong inhibitory effect on viral replication (data not shown); therefore, we next attempted to modify KYK02 to improve its inhibitory effect on viral replication. To this end, we introduced a few additional amino acid substitutions to improve hydrophobic interaction between C34-derived peptide and N-HR, especially in the amino acid residues whose role in inhibiting viral replication has not been sufficiently studied, and generated a series of KYK02-derived peptides (Table 3). These KYK02-derived peptides contained an amino acid substitution, S138A, which frequently appears in the C-HR of T-20-resistant variants (Xu et al., 2005) and was recently revealed to improve binding stability between C34 and N-HR (Izumi et al., 2009; Watabe et al., 2009). The results of neutralization tests showed that five peptides, KYK02-2, KYK02-4, KYK02-6, KYK02-7 and KYK02-9, efficiently inhibited the replication of pNL-T20R (Fig. 2), while four other KYK02-derived peptides could not inhibit its replication (data

**Table 3**
Amino acid sequence of KYK01- or KYK02-derived peptides.

| | 117 150[a] |
|---|---|
| C34 | WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL |
| KYK01-1 | ....EK..D...N..YN.L........E......GGDDDDD[b] |
| KYK01-2 | .E..EKR.D...R..YN.L....R...R..R..K |
| KYK02-1 | .I..E...DR..KE..R.L....RLLDR..KD.K |
| KYK02-2 | .I..E...DR..KE..R.L..A.R..DR..KD.K |
| KYK02-3 | .I..E...DR..KE..R.L..**A**.R..DR..KD.K[c] |
| KYK02-4 | .I..E...DR.VKE..R.L..A.R..DR..KD.K |
| KYK02-5 | .I..E...DR..KE..RAL..A.R..DR..KD.K |
| KYK02-6 | .I..E...DR..KE..R.L..A.RL.DR..KD.K |
| KYK02-7 | .I..E...DR..KE..R.L..A.R.LDR..KD.K |
| KYK02-8 | .I..E...DR.AKE..RAL..A.R..DR..KD.K |
| KYK02-9 | .I..E...DRA.KE..R.L..A.R..DR..KD.K |

[a]Amino acid residues were numbered according to the pNL4-3 gp41 sequence.
[b]Amino acid sequence of KYK01- or KYK02-derived peptides was aligned with that of C34. Dots denote amino acid identity.
[c]D-Alanine residue is shown in bold.

not shown). We next examined the inhibitory effect of five KYK02-derived peptides on the replication of recombinant viruses containing AE-Env, B-Env or C-Env. The results showed that four peptides, KYK02-2, KYK02-4, KYK02-6 and KYK02-9, but not KYK02-7, inhibited the replication of most recombinant viruses more efficiently than T-20 (Fig. 2). In particular, KYK02-2, KYK02-4 and KYK02-6 uniformly inhibited the replication of all recombinant viruses, including pNL-T20R (Fig. 2). Dose-dependent inhibition of pNL-T20R replication by KYK02-2, KYK02-4 and KYK02-6, but not by T-20, is shown in Fig. 3.

*Inhibition of HIV-1 Env-mediated cell fusion by C34-derived peptide, KYK02-4*

We next confirmed that a newly designed C34-derived peptide, KYK02-4, inhibited viral replication by suppressing the viral fusion process. For this purpose, a co-culture experiment was carried out using TZM-bl reporter cells (Derdeyn et al., 2000; Platt et al., 1998; Wei et al., 2002) and 293T cells transfected with the expression vectors for HIV-1 Env and Tat. TZM-bl cells express CD4 and co-receptors, CCR5 and CXCR4, on the cell surface, and also contain the HIV-1 promoter-dependent luciferase gene; thus, luciferase activity is expected to be enhanced if HIV-1 Env-mediated cell fusion occurs between TZM-bl cells and 293T cells expressing HIV-1 Env and Tat.

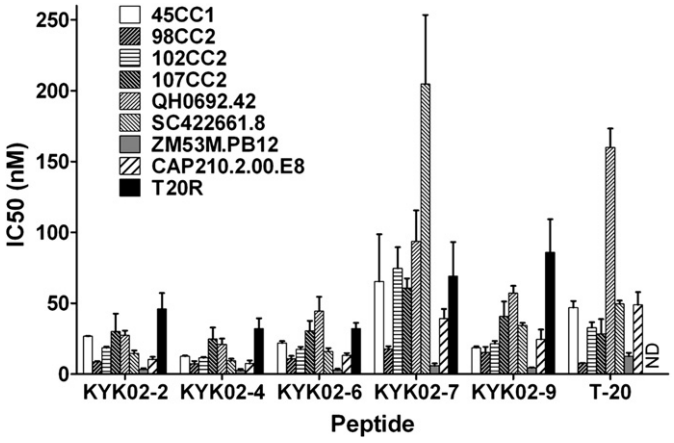


**Fig. 2.** Three KYK02-derived peptides, KYK02-2, KYK02-4 and KYK02-6, efficiently inhibit the replication of all recombinant viruses, including a T-20-resistant virus. The IC50 value of the peptides for inhibiting the replication of the recombinant viruses was calculated as described in the legend to Fig. 1. The T-20-resistant virus, pNL-T20R (T20R), was efficiently replicated in the presence of 5 μM T-20; thus, the IC50 value of T-20 for inhibiting the replication of pNL-T20R was not determined (ND). Data are presented as the means and standard errors (error bars) of three independent experiments.

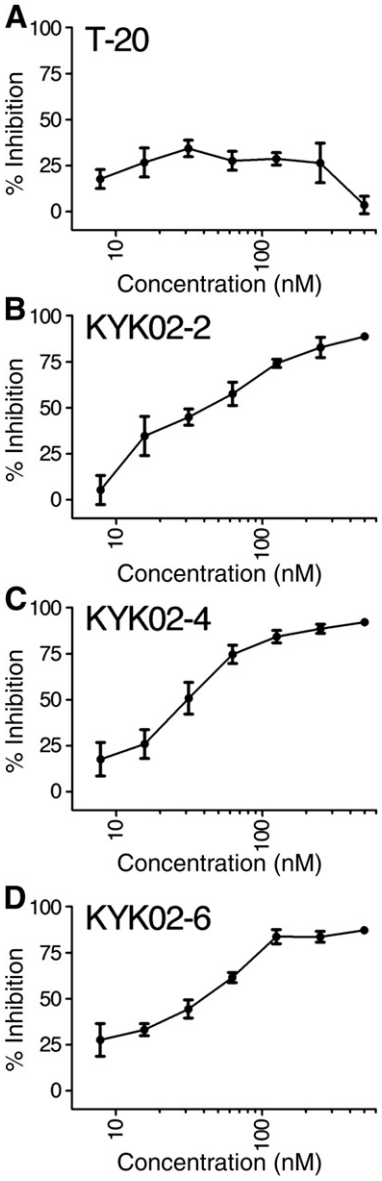


**Fig. 3.** KYK02-2, KYK02-4 and KYK02-6, but not T-20, inhibit the replication of pNL-T20R in a dose-dependent manner. Neutralization susceptibility of pNL-T20R to T-20 (A), KYK02-2 (B), KYK02-4 (C) and KYK02-6 (D) was studied, and the dose–response curves of the inhibition of viral replication by those peptides were generated using GraphPad Prism 5 software, as described in Materials and methods.

The results of a co-culture experiment showed that luciferase activity was significantly enhanced by co-culturing TZM-bl cells with 293T cells transfected with the expression vector for AE-Env, 98CC2 or pNL-T20R Env (T20R-Env) together with the vector for Tat (Fig. 4). In addition, KYK02-4 almost completely prevented such enhancement of luciferase activity (Fig. 4), indicating that cell fusion between TZM-bl cells and 293T cells expressing HIV-1 Env and Tat was inhibited by KYK02-4. In contrast, T-20 failed to prevent the enhancement of luciferase activity in co-culture between TZM-bl cells and 293T cells expressing T20R-Env, as expected (Fig. 4). These results demonstrate that a C34-derived peptide, KYK02-4, inhibited viral replication by suppressing HIV-1 Env-mediated membrane fusion.

*KYK02-4, but not C34, interacts with N36 corresponding to N-HR of pNL-T20R*

We next compared the binding efficiency of C34 and KYK02-4 to the N36 peptide corresponding to N-HR of pNL4-3 (N36-wt) and pNL-

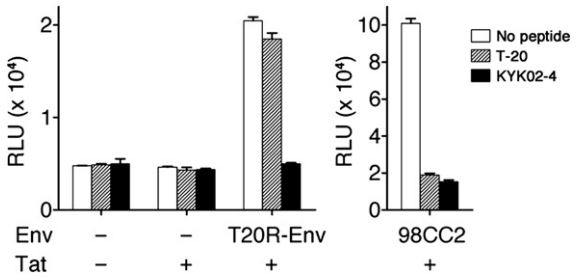


**Fig. 4.** KYK02-4 efficiently inhibits cell fusion mediated by T20R-Env and AE-Env, 98CC2, while T-20 fails to inhibit T20R-Env-mediated cell fusion. 293T cells were transfected with the expression vectors for HIV-1 Tat and Env. Twenty-four hours later, the transfected 293T cells were mock-treated (No peptide) or treated with the indicated peptide (500 nM), and were then co-cultured with TZM-bl cells for 6 h, as described in Materials and methods. Cell fusion was evaluated by measuring luciferase activity (RLU, relative light unit) in TZM-bl cells. Data are presented as the means and standard errors (error bars) of four independent experiments.

T20R (N36-T20R) by *in vitro* reaction followed by polyacrylamide gel electrophoresis under non-denaturing conditions (Native-PAGE), as described previously (He et al., 2008b; Liu et al., 2003). In addition, the heat stability of the complex was examined. The results showed that both C34 and KYK02-4 interacted efficiently with N36-wt (lane 3), and the complexes were quite heat stable, at least under our experimental conditions (lanes 4–7) (Fig. 5). In stark contrast, KYK02-4, but not C36, was able to interact with N36-T20R, although the binding efficiency of KYK02-4 to N36-T20R was lower than that to N36-wt (Fig. 5). These results suggest that KYK02-4, but not C34, is able to interfere with the formation of the 6-helix bundle in infection with not only a wild-type virus, but also a T-20 resistant virus.

*Structural modeling of the interaction between C34-derived peptide and N-HR of pNL-T20R*

Although the C34-derived peptide, KYK02, contained 15 amino acid substitutions, which were expected to improve hydrophobic interaction between N-HR and the peptide (Kawashita, N. et al, submitted), as compared to the original C34, it could not suppress the replication of a T-20-resistant variant of pNL4-3, pNL-T20R (data not shown). In contrast, KYK02-2, KYK02-4 and KYK02-6 effectively inhibited the replication of all Env-recombinant viruses tested, including pNL-T20R (Fig. 2). We next performed structural modeling to study how the additionally introduced amino acid substitutions improved the interaction of N-HR with KYK02-2, KYK02-4 and KYK02-6. These peptides contained the amino acid substitution, S138A, which was already reported to improve binding stability between C34 and N-HR (Izumi et al., 2009; Watabe et al., 2009). KYK02-2, KYK02-4 and KYK02-6, but not KYK02, inhibited the replication of pNL-T20R (Figs. 2 and 3, and data not shown), suggesting that S138A plays a crucial role in the inhibitory effect of these peptides on replication of the T-20-resistant virus. In addition, KYK02-4 and KYK02-6 contained 2 additional amino acid substitutions, T128V and Q141L, as compared to KYK02-2, respectively. KYK02-4 and KYK02-6 showed a stronger inhibitory effect than KYK02-2 on the replication of several Env-recombinant viruses tested (Fig. 2); thus, the amino acid substitutions, T128V and Q141L, may play a role in improving the hydrophobic interaction between the peptide and N-HR of pNL-T20R as well as wild-type viruses. The results of structural modeling showed that T128V and Q141L could be predicted to improve hydrophobic interaction between N-HR and the peptide (Fig. 6). In addition, the amino acid substitution, L150K, which was included in KYK02, KYK02-2, KYK02-4 and KYK02-6, was predicted to improve the interaction between N-HR and the peptide (Fig. 6). These results suggested that multiple amino acid substitutions introduced into the peptides contributed to improve the interaction between the peptide and N-HR, and enhanced the anti-HIV-1 activity of these C34-derived peptides.

Recently, several newly designed peptides which mimic C-HR were reported to efficiently inhibit the replication of T-20-resistant variants derived from subtype B laboratory strains, including pNL4-3 (Dwyer et al., 2007; He et al., 2008a,b; Izumi et al., 2009; Oishi et al., 2008). In addition, some of these peptides were able to broadly inhibit the replication of primary HIV-1 isolates of various subtypes (He et al., 2008a,b). Newly designed C34-derived peptides, KYK02-2, KYK02-4 and KYK02-6, had different amino acid sequences from the previously reported peptides, but were able to inhibit the replication of a T-20-resistant variant of pNL4-3 as well as of recombinant viruses containing subtype B, subtype C or CRF01_AE Env more efficiently than T-20 (Fig. 2); thus, we believe that our study may provide important information to develop novel antiretroviral compounds.

## Materials and methods

*Peptides*

We calculated the stable conformation of the 6-helix bundle, consisting of N-HR and C-HR, by molecular dynamics analysis (Kawashita, N. et al., submitted), and designed several C34-derived peptides, which were expected to interact with N-HR more strongly than the original C34 peptide (Tables 1 and 3). We found that several hydrophobic amino acid residues of C34 play a role in interacting with N-HR (Kawashita, N. et al., submitted); therefore, we designed new

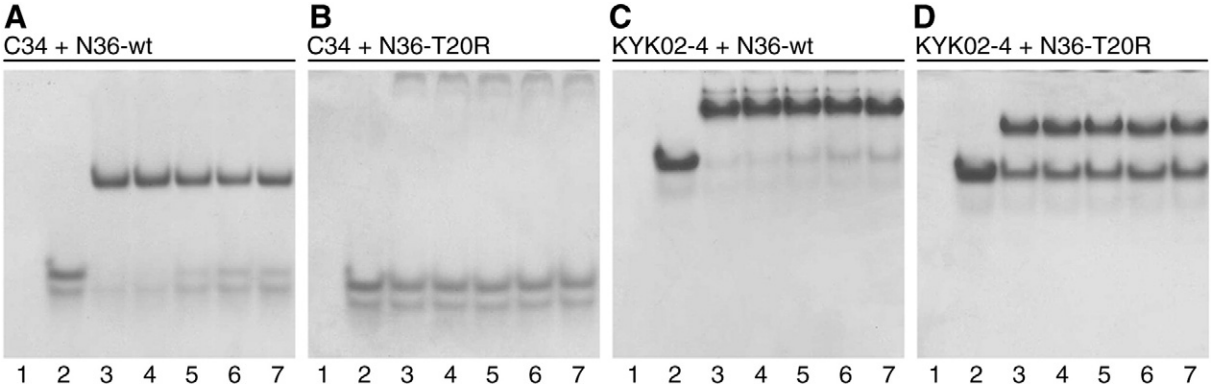


**Fig. 5.** KYK02-4, but not C36, interacts with N36 corresponding to N-HR of pNL-T20R. C34 (A and B) or KYK02-4 (C and D) (1 nmol) was incubated with the N36 peptide corresponding to N-HR of pNL4-3 (N36-wt) (A and C) or pNL-T20R (N36-T20R) (B and D) (1 nmol) for 30 min at 37 °C (lanes 3–7). Samples were then kept on ice (lane 3) or further incubated at 50 °C (lane 4), 60 °C (lane 5), 70 °C (lane 6) or 80 °C (lane 7) for 15 min. Native-PAGE analysis was then carried out as described in Materials and methods. In addition, 1 nmol of N36-wt (A and C, lane 1), N36-T20R (B and D, lane 1), C34 (A and B, lane 2) and KYK02-4 (C and D, lane 2) were subjected to Native-PAGE analysis as control experiments. N36-wt and N36-T20R (lane 1) exhibited no band, because these peptides carry net positive charges and may migrate up and off the gel.

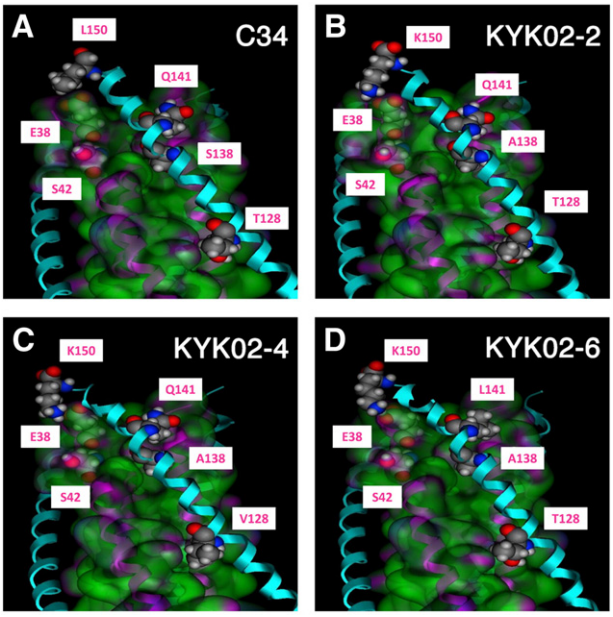


**Fig. 6.** Structural modeling of the interaction between newly designed peptide and N-HR of pNL-T20R. Structural modeling of the interaction of N-HR with C34 (A), KYK02-2 (B), KYK02-4 (C) or KYK02-6 (D) was performed as described in Materials and methods. The locations of amino acid residues, E38 and S42 in N-HR (A, B, C and D, purple), T128, S138, Q141 and L150 in C34 (A, cyan), T128, A138, Q141 and K150 in KYK02-2 (B, cyan), V128, A138, Q141 and K150 in KYK02-4 (C, cyan) and T128, A138, L141 and K150 in KYK02-6 (D, cyan) are shown. Blue, red, white and grey cubes represent nitrogen, oxygen, hydrogen and carbon atoms, respectively. In addition, *van der Waals* surfaces of N-HR are shown in green. The side chain of the amino acid residue, K150 (B, C and D), shows closer proximity to N-HR than L150 (A). In addition, V128 (C) and L141 (D), which lack a hydrophilic hydroxyl group relative to T128 and Q141, are predicted to show tighter hydrophobic interaction with corresponding residues in N-HR, as compared to T128 (A, B and D) and Q141 (A, B and C), respectively.

peptides by replacing non-hydrophobic residues with hydrophobic residues to strengthen the interaction between C34 and N-HR. Synthetic C34-derived peptides were purchased from Biologica, Co. (Nagoya, Japan), dissolved at concentrations of 1–5 mg/ml in 50% dimethyl sulfoxide and stored as aliquots at −85 °C. T-20 was obtained from Roche (Basel, Switzerland) through the AIDS Research and Reference Reagent Program (ARRRP) (Division of AIDS, NIAID, NIH), dissolved at the concentration of 1 mg/ml in phosphate-buffered saline, and stored as aliquots at −85 °C.

### *Cells*

293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (10% FBS-DMEM). TZM-bl cells were obtained from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. through the ARRRP, and were maintained in 10% FBS-DMEM. In addition, U87.CD4.CCR5 and U87.CD4.CXCR4 cells (Bjorndal et al., 1997) were obtained from Dr. HongKui Deng and Dr. Dan R. Littman through the ARRRP, and were maintained in 10% FBS-DMEM with puromycin (1 μg/ml) and G418 (300 μg/ml) (complete medium).

### *Viral constructs*

AE-Env-recombinant proviral constructs containing the CRF01_AE *env* genes, 22PL1, 29CC1, 45CC1, 98CC2 102CC2 and 107CC2, were generated as described previously (Utachee et al., 2009). A T-20-resistant variant of pNL4-3, pNL-T20R, was generated by introducing two amino acid substitutions, V38E and N42S, into the pNL4-3-derived luciferase reporter proviral DNA, pNL-envCT (Kinomoto et al., 2005), by site-directed mutagenesis using the QuikChange® site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). The expression vectors for B-Env clones, QH0692.42, TRO.11, AC10.0.29, PREJO4541.67, pCAAN5342.A2 and SC422661.8 (Li et al., 2005; Wei et al., 2002), and the vectors for C-Env clones, ZM249M.PL1, ZM53M.PB12, ZM109F.PB4, ZM135M.PL10a, CAP45.2.00.G3 and CAP210.2.00.E8 (Li et al., 2006), were obtained from Drs. Cynthia A. Derdeyn, Feng Gao, Beatrice H. Hahn, Eric Hunter, Denise L. Kothe, Ming Li, Yingying Li, Koleka Milsana, David C. Montefiori, Lynn Morris and Jesus F. Salazar-Gonzalez through the ARRRP. Env-deficient, luciferase reporter proviral construct, pNL-Luc-E$^-$R$^+$ (Tokunaga et al., 2001), was used to generate B-Env- and C-Env-recombinant viruses. AE-Env-recombinant viruses, pNL-T20R and pNL-Luc-E$^-$R$^+$, bear the firefly luciferase gene in place of the viral *nef* gene; thus, we could monitor the level of viral replication by measuring luciferase activity in infected cells. In addition, the expression vectors for AE-Env, 98CC2 and pNL-T20R Env (T20R-Env), were generated by introducing *Sal*I–*Not*I fragment, which encodes Env and Rev, of Env-recombinant proviral constructs into the mammalian expression vector, pCI-neo (Promega, Madison, WI).

### *Preparation of Env-recombinant viruses*

293T cells ($2\times10^5$ cells/2 ml) were seeded onto a collagen-coated 6-well plate (Iwaki, Tokyo, Japan) 24 h prior to transfection. AE-Env-recombinant viruses and pNL-T20R were prepared by transfecting 293T cells with the proviral construct (2 μg) using FuGENE HD transfection reagent (Roche). In addition, B-Env- and C-Env-recombinant viruses were prepared by transfecting 293T cells with pNL-Luc-E$^-$R$^+$ (1.33 μg) and the Env-expression vector (0.67 μg). Forty-eight hours after transfection, viral supernatants were cleared by centrifugation for 5 min at 8000 rpm, and stored as aliquots at −85 °C. The viral titer was determined by measuring the concentration of HIV-1 Gag p24 antigen in viral supernatants by enzyme-linked immunosorbent assay (ELISA) (Vironostika HIV-1 Antigen Microelisa System; bioMérieux, Boxtel, The Netherlands).

### *Neutralization tests*

The neutralization susceptibility of Env-recombinant viruses to the peptides was examined as follows. U87.CD4.CXCR4 or U87.CD4.CCR5 cells were incubated with 2-fold serially diluted peptide (concentrations varied depending on the susceptibility of the viruses, ranging from 1 nM to 5 μM) in 100 μl complete medium for 10 min at 37 °C. U87.CD4.CXCR4 cells were used as target cells for recombinant viruses containing CXCR4-tropic AE-Env, 107CC2, and dual-tropic AE-Env, 29CC1 and 45CC1. In addition, U87.CD4.CCR5 cells were used as target cells for recombinant viruses containing CCR5-tropic AE-Env, 22PL1 and 102CC2, six C-Env and six B-Env. The cells were then incubated with viral supernatants (4 ng of p24 antigen) for 48 h. Luciferase activity in infected cells was then measured using the Steady Glo Luciferase assay kit (Promega) with an LB960 microplate luminometer (Berthold, Bad Wildbad, Germany). The inhibitory effect of the peptide on viral replication was evaluated as a reduction in luciferase activity in infected cells. The 50% inhibitory concentration (IC50) of the peptide for inhibiting viral replication was calculated by the dose–response curve using a standard function of GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

### *Cell fusion assay*

293T cells ($2\times10^5$ cells/2 ml) were transfected with the HIV-1 Tat-expression vector, pRSV-Tat (1 μg), together with the expression vector for the AE-Env, 98CC2 or T20R-Env (1 μg), in collagen-coated 6-well plates, essentially as described above. For control experiments, Tat and Env-expression vectors were replaced with the corresponding empty vectors, pRc/RSV (Invitrogen) and pCI-neo, respectively.

Sixteen hours after transfection, the cells were trypsinized and split into six subcultures in a 24-well plate. Twenty-four hours later, the transfected 293T cells were treated with the peptide (500 nM), and then mixed with TZM-bl cells ($6\times10^4$ cells). After 6-h co-culture, the cells were lysed, and luciferase activity in the sample was measured as described above.

### *Native-PAGE analysis for the interaction between N36 and C34-derived peptides*

The N36 peptide corresponding to N-HR of pNL4-3 (N36-wt) or pNL-T20R (N36-T20R) (1 nmol) was mixed with C34 or KYK02-4 (1 nmol) in 16 μl PBS on ice, and the mixture was then incubated for 30 min at 37 °C. Some samples were then further incubated for 15 min at 50–80 °C. The samples were mixed with 4 μl of 5× sample buffer (500 mM Tris–HCl, pH 8.8, 50% glycerol and 0.01% bromophenol blue), and were loaded onto 20% polyacrylamide Tris–glycine gel. Gel electrophoresis was carried out under non-denaturing conditions in 25 mM Tris–192 mM glycine buffer with 100 V of constant voltage for 3 h at 4 °C. The gel was then stained with Coomassie brilliant blue G-250.

### *Structure modeling of the interaction between newly designed peptide and N-HR of the pNL4-3-derived T-20-resistant variant*

The structure of the 6-bundle helix, consisting of N-HR and C-HR (C34), was obtained from X-ray crystallography of N36/C34 complex (PDB identifier: 1aik) (Chan et al., 1997). All model modifications were carried out using MOE (Molecular Operating Environment; Chemical Computing Group Inc., ver. 2008.10), a graphics package for molecular structure analysis. Water molecules were removed from X-ray data, and two amino acid residues of N-HR, V38 and N42, were then replaced with those of the T-20-resistant virus, E38 and S42. Complex models of N-HR/KYK02-2, N-HR/KYK02-4 and N-HR/KYK02-6 were generated from that of N-HR/C34, using the Mutate Prompt module. The first and last residues of each peptide were capped with ACE and NME, respectively, and then hydrogen atoms and partial charges were placed in the optimal positions. Subsequently, ionization states and hydrogen positions were adjusted using the Protonate 3D module. Furthermore, all models were optimized by minimization with the AMBER9 program (Case et al., 2005) using the FF99 force field.

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## References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59, 284–291.

Bjorndal, A., Deng, H., Jansson, M., Fiore, J.R., Colognesi, C., Karlsson, A., Albert, J., Scarlatti, G., Littman, D.R., Fenyo, E.M., 1997. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J. Virol. 71, 7478–7487.

Case, D.A., Cheatham III, T.E., Darden, T., Gohlke, H., Luo, R., Merz Jr., K.M., Onufriev, A., Simmerling, C., Wang, B., Woods, R.J., 2005. The Amber biomolecular simulation programs. J. Comput. Chem. 26, 1668–1688.

Chan, D.C., Chutkowski, C.T., Kim, P.S., 1998. 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, 15613–15617.

Chan, D.C., Fass, D., Berger, J.M., Kim, P.S., 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89, 263–273.

Chan, D.C., Kim, P.S., 1998. HIV entry and its inhibition. Cell 93, 681–684.

Derdeyn, C.A., Decker, J.M., Sfakianos, J.N., Wu, X., O'Brien, W.A., Ratner, L., Kappes, J.C., Shaw, G.M., Hunter, E., 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. 74, 8358–8367.

Dwyer, J.J., Wilson, K.L., Davison, D.K., Freel, S.A., Seedorff, J.E., Wring, S.A., Tvermoes, N. A., Matthews, T.J., Greenberg, M.L., Delmedico, M.K., 2007. Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. Proc. Natl. Acad. Sci. U. S. A. 104, 12772–12777.

Gaschen, B., Taylor, J., Yusim, K., Foley, B., Gao, F., Lang, D., Novitsky, V., Haynes, B., Hahn, B.H., Bhattacharya, T., Korber, B., 2002. Diversity considerations in HIV-1 vaccine selection. Science 296, 2354–2360.

Greenberg, M.L., Cammack, N., 2004. Resistance to enfuvirtide, the first HIV fusion inhibitor. J. Antimicrob. Chemother. 54, 333–340.

He, Y., Cheng, J., Lu, H., Li, J., Hu, J., Qi, Z., Liu, Z., Jiang, S., Dai, Q., 2008a. Potent HIV fusion inhibitors against Enfuvirtide-resistant HIV-1 strains. Proc. Natl. Acad. Sci. U. S. A. 105, 16332–16337.

He, Y., Xiao, Y., Song, H., Liang, Q., Ju, D., Chen, X., Lu, H., Jing, W., Jiang, S., Zhang, L., 2008b. Design and evaluation of sifuvirtide, a novel HIV-1 fusion inhibitor. J. Biol. Chem. 283, 11126–11134.

Hemelaar, J., Gouws, E., Ghys, P.D., Osmanov, S., 2006. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS 20, W13–W23.

Izumi, K., Kodama, E., Shimura, K., Sakagami, Y., Watanabe, K., Ito, S., Watabe, T., Terakawa, Y., Nishikawa, H., Sarafianos, S.G., Kitaura, K., Oishi, S., Fujii, N., Matsuoka, M., 2009. Design of peptide-based inhibitors for Human Immunodeficiency Virus Type 1 strains resistant to T-20. J. Biol. Chem. 284, 4914–4920.

Kilby, J.M., Hopkins, S., Venetta, T.M., DiMassimo, B., Cloud, G.A., Lee, J.Y., Alldredge, L., Hunter, E., Lambert, D., Bolognesi, D., Matthews, T., Johnson, M.R., Nowak, M. A., Shaw, G.M., Saag, M.S., 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. Nat. Med. 4, 1302–1307.

Kinomoto, M., Yokoyama, M., Sato, H., Kojima, A., Kurata, T., Ikuta, K., Sata, T., Tokunaga, K., 2005. Amino acid 36 in the human immunodeficiency virus type 1 gp41 ectodomain controls fusogenic activity: implications for the molecular mechanism of viral escape from a fusion inhibitor. J. Virol. 79, 5996–6004.

Kliger, Y., Gallo, S.A., Peisajovich, S.G., Munoz-Barroso, I., Avkin, S., Blumenthal, R., Shai, Y., 2001. Mode of action of an antiviral peptide from HIV-1. Inhibition at a post-lipid mixing stage. J. Biol. Chem. 276, 1391–1397.

Lalezari, J.P., Henry, K., O'Hearn, M., Montaner, J.S., Piliero, P.J., Trottier, B., Walmsley, S., Cohen, C., Kuritzkes, D.R., Eron Jr., J.J., Chung, J., DeMasi, R., Donatacci, L., Drobnes, C., Delehanty, J., Salgo, M., 2003. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. N. Engl. J. Med. 348, 2175–2185.

Lazzarin, A., Clotet, B., Cooper, D., Reynes, J., Arasteh, K., Nelson, M., Katlama, C., Stellbrink, H.J., Delfraissy, J.F., Lange, J., Huson, L., DeMasi, R., Wat, C., Delehanty, J., Drobnes, C., Salgo, M., 2003. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. N. Engl. J. Med. 348, 2186–2195.

Li, M., Gao, F., Mascola, J.R., Stamatatos, L., Polonis, V.R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P., Greene, K.M., Bilska, M., Kothe, D.L., Salazar-Gonzalez, J. F., Wei, X., Decker, J.M., Hahn, B.H., Montefiori, D.C., 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. 79, 10108–10125.

Li, M., Salazar-Gonzalez, J.F., Derdeyn, C.A., Morris, L., Williamson, C., Robinson, J.E., Decker, J.M., Li, Y., Salazar, M.G., Polonis, V.R., Mlisana, K., Karim, S.A., Hong, K., Greene, K.M., Bilska, M., Zhou, J., Allen, S., Chomba, E., Mulenga, J., Vwalika, C., Gao, F., Zhang, M., Korber, B.T., Hunter, E., Hahn, B.H., Montefiori, D.C., 2006. Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. J. Virol. 80, 11776–11790.

Liu, S., Zhao, Q., Jiang, S., 2003. Determination of the HIV-1 gp41 fusogenic core conformation modeled by synthetic peptides: applicable for identification of HIV-1 fusion inhibitors. Peptides 24, 1303–1313.

Malashkevich, V.N., Chan, D.C., Chutkowski, C.T., Kim, P.S., 1998. Crystal structure of the simian immunodeficiency virus (SIV) gp41 core: conserved helical interactions underlie the broad inhibitory activity of gp41 peptides. Proc. Natl. Acad. Sci. U. S. A. 95, 9134–9139.

McCutchan, F.E., 2006. Global epidemiology of HIV. J. Med. Virol. 78 (Suppl 1), S7–S12.

Oishi, S., Ito, S., Nishikawa, H., Watanabe, K., Tanaka, M., Ohno, H., Izumi, K., Sakagami, Y., Kodama, E., Matsuoka, M., Fujii, N., 2008. Design of a novel HIV-1 fusion inhibitor that displays a minimal interface for binding affinity. J. Med. Chem. 51, 388–391.

Platt, E.J., Wehrly, K., Kuhmann, S.E., Chesebro, B., Kabat, D., 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J. Virol. 72, 2855–2864.

Tokunaga, K., Greenberg, M.L., Morse, M.A., Cumming, R.I., Lyerly, H.K., Cullen, B.R., 2001. Molecular basis for cell tropism of CXCR4-dependent human immunodeficiency virus type 1 isolates. J. Virol. 75, 6776–6785.

Utachee, P., Jinnopat, P., Isarangkura-Na-Ayuthaya, P., de Silva, U.C., Nakamura, S., Siripanyaphinyo, U., Wichukchinda, N., Tokunaga, K., Yasunaga, T., Sawanpanyalert,

P., Ikuta, K., Auwanit, W., Kameoka, M., 2009. Genotypic characterization of CRF01_AE env genes derived from Human Immunodeficiency Virus type 1-infected patients residing in Central Thailand. AIDS Res. Hum. Retroviruses 25, 229–236.
Watabe, T., Terakawa, Y., Watanabe, K., Ohno, H., Nakano, H., Nakatsu, T., Kato, H., Izumi, K., Kodama, E., Matsuoka, M., Kitaura, K., Oishi, S., Fujii, N., 2009. X-ray crystallographic study of an HIV-1 fusion inhibitor with the gp41 S138A substitution. J. Mol. Biol. 392, 657–665.
Wei, X., Decker, J.M., Liu, H., Zhang, Z., Arani, R.B., Kilby, J.M., Saag, M.S., Wu, X., Shaw, G. M., Kappes, J.C., 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. 46, 1896–1905.
Weiss, C.D., 2003. HIV-1 gp41: mediator of fusion and target for inhibition. AIDS Rev. 5, 214–221.
Wild, C., Greenwell, T., Matthews, T., 1993. A synthetic peptide from HIV-1 gp41 is a potent inhibitor of virus-mediated cell–cell fusion. AIDS Res. Hum. Retroviruses 9, 1051–1053.
Wild, C.T., Shugars, D.C., Greenwell, T.K., McDanal, C.B., Matthews, T.J., 1994. 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, 9770–9774.
Wyatt, R., Sodroski, J., 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 280, 1884–1888.
Xu, L., Pozniak, A., Wildfire, A., Stanfield-Oakley, S.A., Mosier, S.M., Ratcliffe, D., Workman, J., Joall, A., Myers, R., Smit, E., Cane, P.A., Greenberg, M.L., Pillay, D., 2005. Emergence and evolution of enfuvirtide resistance following long-term therapy involves heptad repeat 2 mutations within gp41. Antimicrob. Agents Chemother. 49, 1113–1119.