

研究成果の刊行に関する一覧表（総合）

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Characterization of HIV-1 resistance to a fusion inhibitor, N36, derived from the gp41 amino-terminal heptad repeat

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ABSTRACT

A transmembrane glycoprotein of HIV-1, gp41, plays a central role in membrane fusion of HIV-1 and host cells. Peptides derived from the amino- and carboxyl-terminal heptad repeat (N-HR and C-HR, respectively) of gp41 inhibit this fusion. The mechanism of resistance to enfuvirtide, a C-HR-derived peptide, is well defined; however the mechanism of resistance to N-HR-derived peptides remains unclear. We characterized an HIV-1 isolate resistant to the N-HR-derived peptide, N36. This HIV-1 acquired a total of four amino acid substitutions, D36G, N126K and E137Q in gp41, and P183Q in gp120. Among these substitutions, N126K and/or E137Q conferred resistance to not only N36, but also C34, which is the corresponding C-HR-derived peptide fusion inhibitor. We performed crystallographic and biochemical analysis of the 6-helix bundle formed by synthetic gp41-derived peptides containing the N126K/E137Q substitutions. The structure of the 6-helix bundle with N126K/E137Q was identical to that in wild-type HIV-1 except for the presence of a new hydrogen bond. Denaturing experiments revealed that the stability of the 6-helix bundle of N126K/E137Q is greater than in the wild-type. These results suggest that the stabilizing effect of N126K/E137Q provides resistance to N36 and C34.

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1. Introduction

An envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1), gp120, interacts with CD4 and co-receptors, such as CCR5. This induces conformational changes of gp120 to activate gp41, which mediates viral membrane fusion to the host membrane (Eckert and Kim, 2001b). Briefly, after the fusion domain located in the N-terminal end of gp41 penetrates into the host cell

membrane, the interaction of a trimer of the amino-terminal heptad repeat (N-HR) with the carboxyl-terminal HR (C-HR) of gp41, results in the formation of a 6-helix bundle in which the N- and C-HRs are arranged in a three-hairpin structure. Alternatively, the three N-HRs form a coiled-coil, and the three C-HRs are packed in an antiparallel manner into highly conserved, hydrophobic grooves on the surface of the coiled-coil (Chan et al., 1997). Peptides derived from the N- and C-HR regions inhibit fusion by blocking the interaction between the N- and C-HRs and preventing the formation of the 6-helix bundle fusogenic state of gp41 (Chan et al., 1998; Wild et al., 1993, 1992). One of the C-HR-derived peptides, enfuvirtide (T-20), effectively suppresses HIV-1 replication *in vivo* (Kilby et al., 1998; Lalezari et al., 2003; Lazzarin et al., 2003). Another C-HR-derived peptide, C34, which contains the four amino acids, W117, W120, D121, and I124, required to dock into a hydrophobic pocket termed the “deep pocket” of the trimer of the N-HR also exerts strong inhibition of HIV-1 fusion *in vitro* (Chan et al., 1997). In addition to peptides derived from the HIV-1 gp41 consensus amino acid sequence, several modified peptides have also been developed, including T-1249 (Eron et al., 2004), T-2635 (Dwyer et al., 2007), SC34EK (Nishikawa et al., 2009) and T-20_{S138A} (Izumi et al., 2009).

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To date, resistant variants to C-HR-derived peptides, such as T-20 and C34, have been extensively investigated. Resistant variants to T-20 have emerged with mutations in the N-HR region, especially from L33 to L45, which is thought to be the binding site of T-20, both *in vitro* (Fikkert et al., 2002; Rimsky et al., 1998) and *in vivo* (Aquaro et al., 2006; Bienvenu et al., 2006; Cabrera et al., 2006; Labrosse et al., 2006; Menzo et al., 2004; Perez-Alvarez et al., 2006; Ray et al., 2007; Wei et al., 2002), suggesting that substitutions in the N-HR directly interfere with T-20 binding. Variants resistant to C34 also emerged *in vitro* with amino acid mutations in the N-HR region (Armand-Ugon et al., 2003; Lohrengel et al., 2005; Nameki et al., 2005). Taken together, mutations in the N-HR region play a key role in resistance to C-HR-derived peptides.

An N-HR-derived peptide N36 corresponding to the leucine/isoleucine zipper sequence of gp41 has anti-HIV-1 activity to a lesser extent when compared to T-20 and C34 (Dubay et al., 1992; Wild et al., 1994; Wild et al., 1992). It is believed that N36 easily aggregates in physiological solutions, resulting in reduced potency (Lu et al., 1995; Lu and Kim, 1997). Meanwhile, a stabilized and trimeric coiled-coil N peptide, IZN17, was developed and displayed 100-fold greater potency compared with N36 (Eckert and Kim, 2001a). Moreover, (CCIZN17)₃, a covalently stabilized trimer of IZN17s, represented 30-fold increase in potency compared with IZN17 (Bianchi et al., 2005). These studies demonstrated that appropriate engineering of N-HR-derived peptides could increase their inhibitory effect, suggesting that development of N-HR-derived peptides is one of the novel candidates for effective HIV-1 inhibitors.

In this study, we selected for and characterized HIV-1 variants resistant to N36 by virological, biochemical and X-ray crystallographic analyses, with the aim of elucidating further information regarding HIV-1 fusion.

2. Materials and methods

2.1. Cells and viruses

MT-2 and 293T cells were grown in RPMI 1640 medium and Dulbecco's modified Eagle medium (DMEM), respectively. HeLa-CD4-LTR- β -gal cells were kindly provided by M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease (Bethesda, MD, USA), and used for the drug susceptibility assay as described previously (Nameki et al., 2005). Recombinant infectious HIV-1 clones carrying various mutations were generated by pNL4-3 plasmid with site-directed mutagenesis as described previously (Nameki et al., 2005). Each molecular clone was transfected into 293T cells with TransIT[®] (Mirus Bio LLC, Madison, WI, USA). After 48 h, the supernatants were harvested and stored at -80°C until required.

2.2. Antiviral agents

The peptides used in this study were synthesized as described previously (Otaka et al., 2002). A reverse transcriptase inhibitor, 2',3'-dideoxycytidine (ddC), and an adsorption inhibitor, dextran sulfate (DS5000) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Determination of drug susceptibility

The peptide sensitivity of infectious clones was determined by the multinuclear activation of galactosidase indicator (MAGI) assay as described previously (Nameki et al., 2005). Briefly, the target cells (HeLa-CD4-LTR- β -gal; 10^4 cells/well) were plated in 96-well flat-bottomed microtiter culture plates. On the following

day, the cells were inoculated with the HIV-1 clones (60 MAGI unit/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. Forty-eight hours after viral exposure, cells were stained with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and blue cells were counted in each well. The activity of test compounds was determined as the effective concentration that blocked HIV-1 replication by 50% (EC_{50}).

2.4. Induction of HIV-1 variants resistant to N36

MT-2 cells were exposed to wild-type HIV-1 (HIV-1_{WT}) and cultured in the presence of N36 at an initial concentration of 0.1 μM . Cultures were incubated at 37°C until extensive cytopathic effect (CPE) was observed. The culture supernatants were used for further passage of MT-2 cells in the presence of increasing concentrations of N36 until massive CPE was seen at earlier stages of culture. Such dose-escalating culture was performed until resistant variants were obtained. This selection was carried out for a total of 25 passages. At the indicated passages, 10, 20, and 22, the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from the infected MT-2 cells.

2.5. Viral replication kinetics assay

MT-2 cells (10^5 cells/mL) were infected with each virus preparation (500 MAGI unit) for 16 h. The infected cells were then washed and cultured in a final volume of 3 mL. The culture supernatants were collected from days 2–7 after infection, and the amount of p24 antigen was determined.

2.6. Circular dichroism (CD) spectroscopy

Each peptide was incubated at 37°C for 30 min, with the final concentration of peptides were 10 μM in phosphate buffered saline (PBS) pH 7.4. CD spectra were recorded on an AVIV model 202 spectropolarimeter (AVIV) with a 1 mm path-length cuvette at 25°C from an average of eight scans. The thermal stability was assessed by monitoring the change in the CD signal at 222 nm. The midpoint of the thermal unfolding transition, the melting temperature (T_m) of each complex was determined as described previously (Otaka et al., 2002).

2.7. Crystallization, data collection and refinement

Crystallization was performed using the hanging-drop vapor-diffusion method at 4°C . The solution for crystallization was prepared by mixing 2 μL peptide solution (10 mg/mL each of N36 and C34_{KQ} peptides) with 2 μL of mother liquor. The triangular prism-shaped crystals of the N36/C34_{KQ} complex were grown in 80 mM ammonium chloride, 16% 2-methylpentan-2,4-diol and 25% isopropanol, which diffract to beyond 1.7 Å resolution and belong to space group C2 with unit-cell parameters $a=88.63$, $b=50.48$, $c=56.15$ Å, $\beta=90.88^{\circ}$. X-ray diffraction data were collected at 100 K on a rotating copper-anode home X-ray source (MicroMax-007, Rigaku, Japan) equipped with an imaging plate detector (R -axis IV⁺⁺, Rigaku). The structure was solved by molecular replacement using the program MOLREP (Vagin and Teplyakov, 1997) with the model of a wild-type 6-helical bundle structure which was generated by symmetry operations from the PDB coordinate file 1AIK. Structure refinement was performed with the programs CNS (Brunger et al., 1998) and XtalView (McRee, 1999).

3. Results

3.1. Selection of N36-resistant HIV-1

In order to induce HIV-1 variants resistant to N36, escalating doses of N36 (from 0.1 μM) were applied to HIV-1_{WT}-infected MT-2 cells. At passage 11 (P-11), P-20 and P-22, when the concentration of N36 was 6.4, 12.8 and 25.6 μM , respectively, the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from MT-2 cells as described previously (Fig. 1) (Nameki et al., 2005). Sequence analysis of HIV-1 at P-11 revealed that the aspartic acid residue at position 36 (D36) and the asparagine at position 126 (N126) of gp41 had been substituted for glycine (D36G) and lysine (N126K), respectively. At P-20 and P-22, E137Q in gp41 and P183Q in gp120 had emerged, respectively. Both N126K and E137Q substitutions were located in the C-HR which is thought to be the interactive site of N36 (Fig. 1A) (Chan et al., 1997). An N126K mutation was also induced in resistant viruses to C34 (Nameki et al., 2005), T-20 (Baldwin et al., 2004), modified C-HR-derived peptide, T-1249 (Eggink et al., 2008), and cell membrane-anchored C-peptide, maC46 (Hermann et al., 2009). In the bulk-sequencing, we found mixed substitution at N126K, AAG and AAA. Therefore, we cloned PCR products to a cloning vector pSL301 (Invitrogen, Carlsbad, CA, USA) and revealed that 6 and 3 clones were AAG and AAA, respectively, and that no other substitutions in the both HRs were observed.

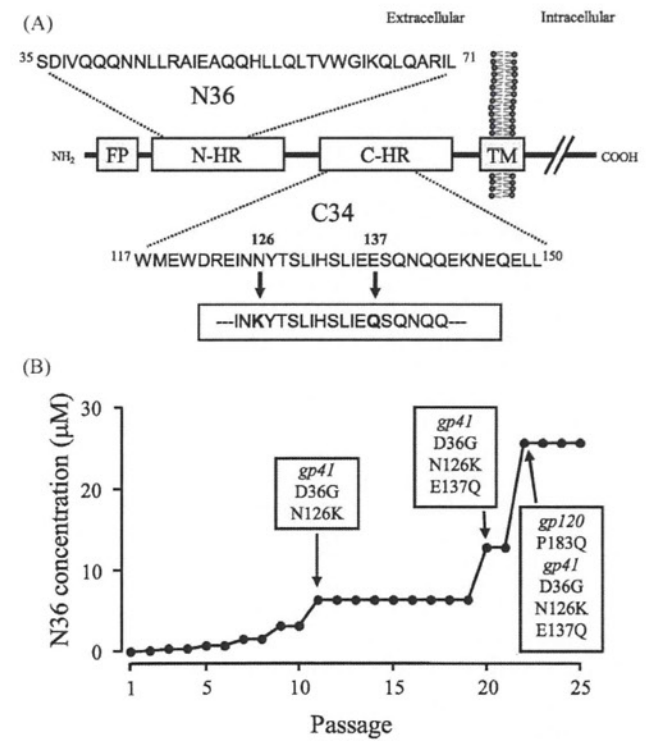


Fig. 1. Schematic view of HIV-1 gp41 (A) and induction of N36-resistant HIV-1 (B). The locations of the fusion peptide (FP), N-terminal heptad repeat region (N-HR), C-terminal heptad repeat region (C-HR), transmembrane domain (TM), and the gp41-derived peptides, N36 and C34, are shown (A). The residue numbers of each peptide correspond to their positions in gp41. The bold underlined letters in the box indicate the novel mutations that were observed in the C-HR of N36-resistant HIV-1 variants. (B) HIV-1_{WT} was passaged in MT-2 cells with increasing concentrations of N36. The dose-escalating selection was carried out for a total of 25 passages, with compound concentrations ranging from 0.1 to 25.6 μM . At the indicated passages, proviral DNA from the lysates of infected cells were sequenced.

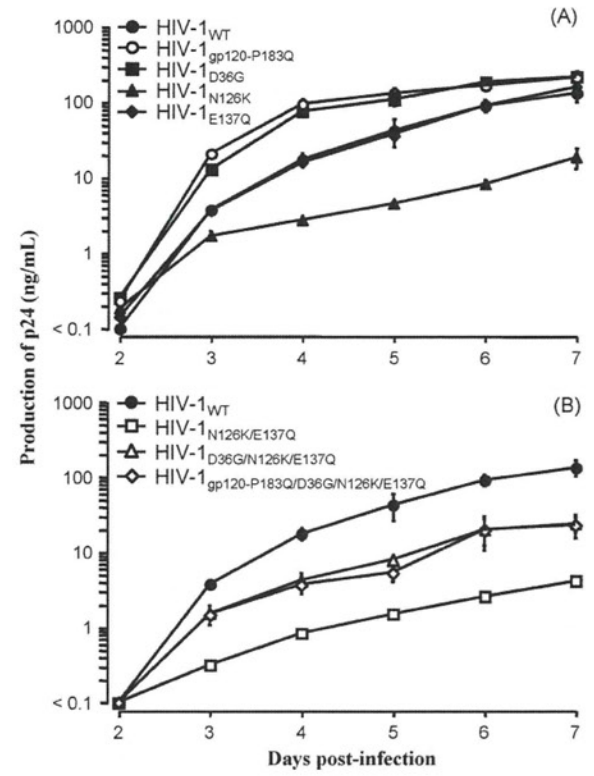


Fig. 2. Replication kinetics of N36-resistant variants. Replication kinetics of N36-resistant HIV-1 variants with a single (A) or combination of mutations (B). Supernatants were collected on days 2–7 from infected MT-2 cells and were subjected to determination of p24 production. Representative results show the mean and standard deviation of experiments performed independently three times.

3.2. Susceptibility of HIV-1 variants to N36

To identify which substitutions were responsible for N36 resistance, we generated seven recombinant viruses, and examined the susceptibility of N36 against these viruses with the MAGI assay. HIV-1_{N126K}, HIV-1_{E137Q}, HIV-1_{N126K/E137Q}, HIV-1_{D36G/N126K/E137Q}, and HIV-1_{gp120-P183Q/D36G/N126K/E137Q} showed reduced susceptibility to N36 (Table 2). Since N126K emerged in HIV-1 variants resistant to C-HR-derived peptides (Baldwin et al., 2004; Nameki et al., 2005), all N126K containing viruses also demonstrated a 4–5-fold reduction in susceptibility to C34. The D36G substitution, observed in the majority of HIV-1 strains (Kuiken et al., 2009), and P183Q which is located in the gp120 V2 region was observed in a wide range of HIV-1 subtypes including subtypes A, B, and C as well as the wild-type, indicating that P183Q is a polymorphism (Kuiken et al., 2009). These results indicate that the single mutations, N126K and E137Q, are involved in resistance to N36, but when both mutations were present, only a weak effect on resistance was observed.

3.3. Replication kinetics of N36-resistant variants

To address effects of the mutations on HIV-1 replication, we examined replication kinetics of HIV-1 variants through p24 production in culture supernatants. The N126K substitution had an adverse effect on replication kinetics, while E137Q exhibited no effect on replication kinetics compared to HIV-1_{WT} (Fig. 2A). The variant, HIV-1_{N126K/E137Q} had markedly reduced replication kinetics, however the D36G mutation moderately restored these kinetics

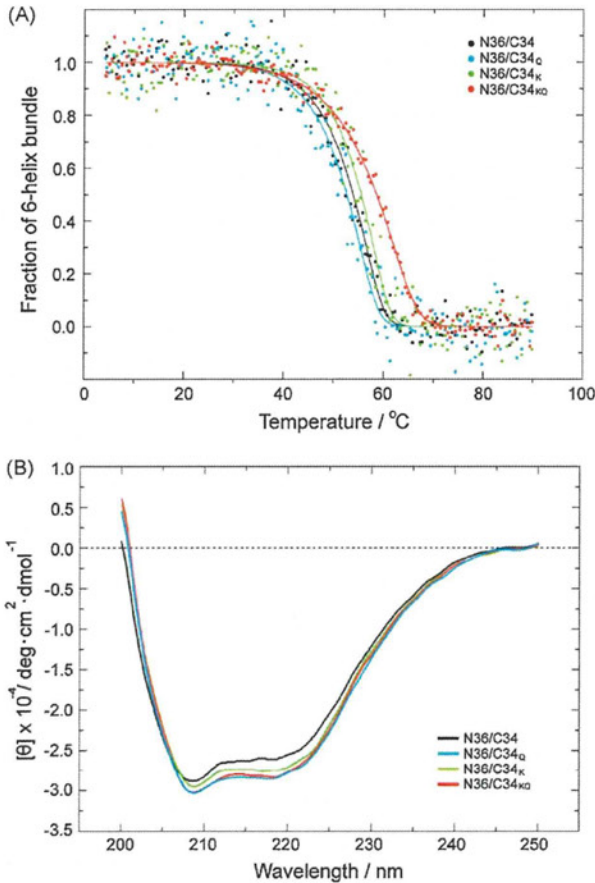


Fig. 3. Affinity of N36 and C34 with or without mutation. Melting temperature (T_m) and CD spectra of the N36/C34 (wild-type), N36/C34_K, N36/C34_Q and N36/C34_{KQ} complexes are shown (A). CD spectra profile of N36/C34 and N36/C34_{KQ} complexes are shown (B).

(Fig. 2B). These results indicate that the primary mutations, N126K and E137Q, are strongly and weakly associated with a reduction in HIV-1 replication kinetics, respectively. In contrast, P183Q in gp120 solely enhanced the replication kinetics of HIV-1 (Fig. 2A) but did little to alter the replication kinetics of HIV-1_{D36G/N126K/E137Q} (Fig. 2B). P183Q and D36G appear to be secondary or spontaneous mutations in HIV-1 induced by serial passage in an attempt to adapt to a cell culture environment.

3.4. Thermal stability of the 6-helix bundle

Synthetic peptides representing the N- and C-HRs have been shown to fold into thermally stable 6-helix bundles (Lu et al., 1999; Lu and Kim, 1997), and thus provide a model of gp41 for investigating structural and other biophysical properties of a 6-helix bundle. To determine whether N126K and E137Q contributed to the stability of the 6-helix bundle, we measured the T_m of the complexes, N36/C34 (wild-type) and N36/C34_{KQ} (C34 with N126K and E137Q mutations). As shown in Fig. 3A, T_m values of the complex of N36/C34_K and N36/C34_Q were similar to that of N36/C34 complex, while that of N36/C34_{KQ} was increased. In addition, the CD spectra observed in N36/C34_{KQ} and N36/C34_Q at 25 °C showed a high helix extent compared to that in N36/C34, while N36/C34_K showed intermediate helicity (Fig. 3B). These results indicate that the two mutations, N126K and E137Q, co-operatively provide high thermal stability of the N36/C34_{KQ} complex.

Table 1

Crystallization data, statistics and refinement parameters.

Crystallization	
Peptide solution	
N36	10 mg/mL
C34 _{KQ}	10 mg/mL
Mother liquor	
Ammonium chloride	80 mM
2-Methylpentan-2,4-diol	16%
Isopropanol	25%
Data collection	
Resolution range/Å	35–1.7
No. of observed reflections	99,054
No. of unique reflections	27,434
Completeness	99.1%
Redundancy	3.61
<i>R</i> _{merge}	7.2%
Space group	C2
Unit-cell parameters	
	$a = 88.63, b = 50.48, c = 56.15 \text{ \AA}, \beta = 90.88^\circ$
Refinement	
Resolution range/Å	23.8–1.7
No. of reflections in the working set	25,818
No. of protein atoms	1803
No. of water molecules	146
<i>R/R</i> _{free}	18.8/21.9%

3.5. Crystal structure of the 6-helix bundle

To reveal the structural basis of the resistance and stabilization mechanisms by the double mutations, we solved the crystal structure of the N36/C34_{KQ} complex (PDB code 3AHA). The statistics for data collection and refinement are summarized in Table 1. The solved structure showed a 6-helix bundle structure, which was the same as in the wild-type N36/C34 complex (PDB code 1AIK) (Fig. 4). Their main chain structures are completely identical in helical pitch as well as in twist angle. A careful comparison between the N36/C34 and N36/C34_{KQ} complexes revealed some differences in side-chain interactions. The N36/C34 complex has been solved in the trigonal *P*321 space group so that the asymmetric unit contains one of each N36 and C34 peptide, in which symmetrical side-chain interactions along the 3-fold rotation axis are formed around the bundle. We solved the N36/C34_{KQ} complex in the lower symmetry C2 space group, and this asymmetric unit contained one helix bundle structure (Fig. 4).

The N126K mutation does not seem to induce conformational changes in the crystal structure when compared to the N36/C34 complex (Fig. 4C). It is consistent with the location of N126 that the N126K mutation does not directly interact with the binding groove formed by the N-HR trimer (Fig. 4A). In the case of E137Q, local rearrangement of a hydrogen bond was induced (Fig. 4D–G), although the hydrogen bond network formed by E/Q137–N43–R46–N42–Q142′–Q40′ is maintained. These asymmetrical and inter-helical side-chain interactions may contribute to the stability of gp41.

4. Discussion

In this study, we induced N36-resistant variants of HIV-1 *in vitro* and characterized them. The variants contained four mutations, P183Q in gp120 and D36G, N126K, and E137Q in gp41. Among these mutations, N126K and E137Q were directly associated with N36 resistance. The N126K mutation also conferred resistance in HIV-1 to C-HR-derived peptides (Baldwin et al., 2004; Eggink et al., 2008; Nameki et al., 2005) and a cell surface expressed peptide (Hermann et al., 2009), indicating that N126K is a key mutation for acquisition

of resistance to all gp41-derived fusion inhibitors.

E137Q was not observed as a polymorphism (Kuiken et al., 2009) and was not identified in HIV-1 that was resistant to the NHR-derived peptide, N13ΔTrimer, which had reasonable solubility, high helicity and thermostability (Dwyer et al., 2008). Therefore, E137Q is a novel mutation for N36 resistance. The solved crystal structure revealed that E137Q induces local rearrangement of the hydrogen bond network in gp41. A polymorphism E137K (Kuiken et al., 2009) has been identified as one of resistance associated mutations to N44 in a CCR5 tropic HIV strain, HIV-1_{JR-CSF} (Desmezieres et al., 2005). Recently, Tolstrup et al. (2007) also reported that E137K restored T-20-resistant virus infectivity impaired by the acquisition of the N43D mutation. Since E137 formed hydrogen bonds with N43, it is likely that the E137K mutation partially restores the 6-helix bundle stability (Bai et al., 2008), suggesting that E137 is an important position for stability of the 6-helix bundle and E137Q

is a mutation for conferring N36-resistance in HIV-1. On the other hand, the overall structure of the 6-helix bundle with mutations was barely affected, which is consistent with our recent observations. The C34 derivative, SC34EK, which contains 12 hydrophilic and one artificial substitution (Nishikawa et al., 2009) has highly potent anti-HIV activity and maintains its structure in the 6-helix bundle even with these extensive modifications. These results indicate that the basic structure of the 6-helix bundle appears to be crucial for gp41 function, suggesting that agents which disrupt this structure will have inhibitory effects upon fusion.

Other substitutions observed in the N36 selection, P183Q in gp120 and D36G in gp41, enhanced viral replication kinetics (Fig. 2) but little influenced N36 susceptibility (Table 2). It is likely that faster entry kinetics theoretically provides resistance to fusion inhibitors through relatively short period to allow interaction of fusion inhibitors with the target, gp41. Indeed, HIV-1 with the

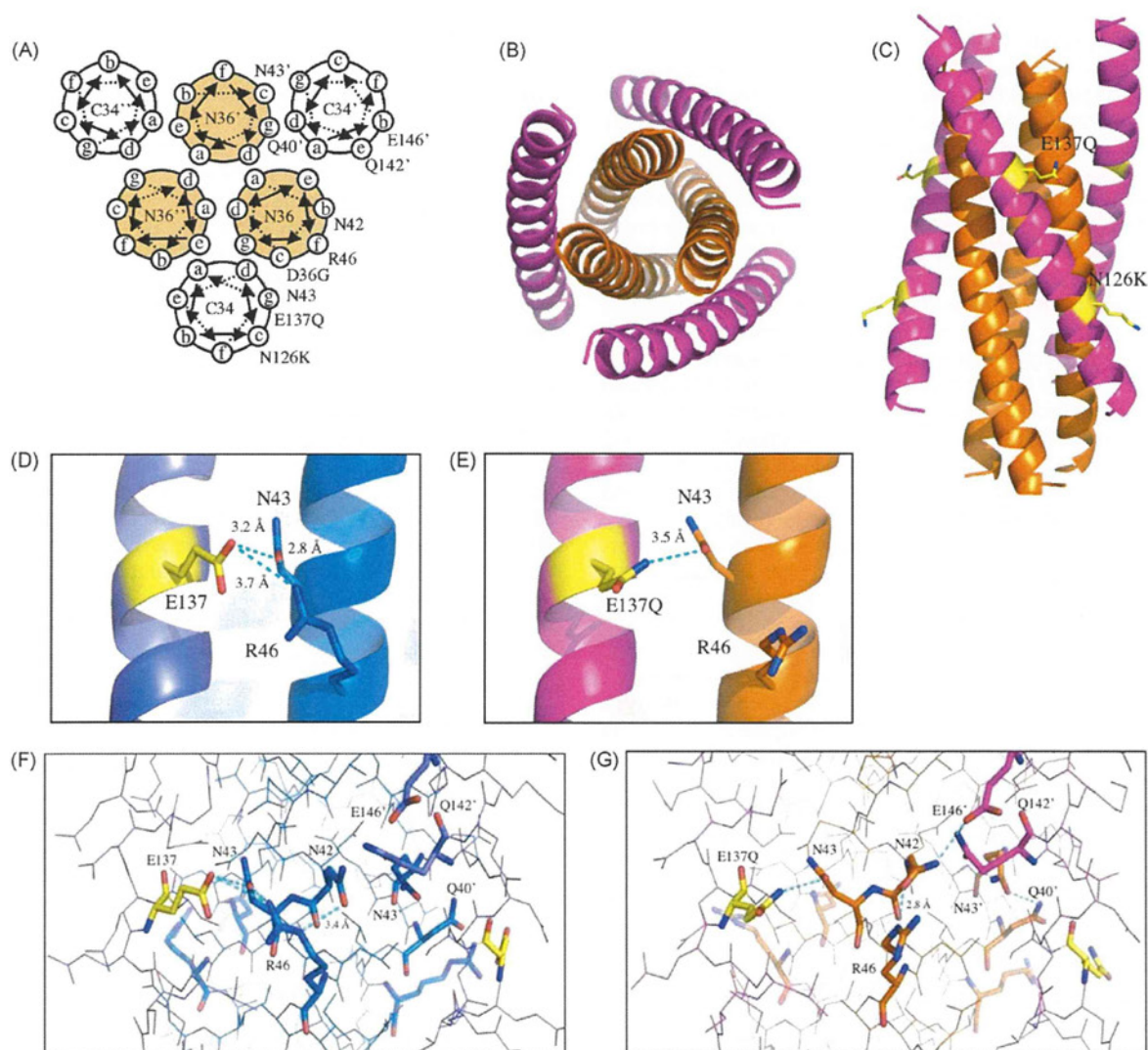


Fig. 4. Helical wheel representation of N36 and C34, and structure of N36-resistant HIV-1 gp41. The 6-helix bundle is represented as helical wheel projections (Chan et al., 1997) and the view is from the top of the complex. N36 and C34, N36' and C34', and N36'' and C34'' indicate each pair of a N36- and a C34-helix. The position of amino acid mutations in N36-resistant HIV-1 and the amino acids related to formation of hydrogen bonds is shown (A and B). (C) The entire structure of the 6-helix bundle with N126K and E137Q mutations. The views, focused on binding between an N36 and a C34 (D and F) and an N36 and a C34_{KQ} (E and G), are shown. A hydrogen bond is depicted by the blue dashed line. (D) A hydroxyl group of the E137 side chain formed two hydrogen bonds with the amide oxygen of N43 and the amine of R46. A hydrogen bond also formed between the amide oxygen of N43 and the amine of R46. (E) The amide hydrogen of the E137Q side chain sustains a hydrogen bond with the amide oxygen of N43. The hydrogen bond formed between the amide hydrogen of E137Q and the amine of R46 was lost. The hydrogen bond formed between the amide oxygen of N43 and the amine of R46 in N36 was also lost. The views, focused on the hydrogen bond in the entire 6-helix bundle, formed with N36 and C34 (F) and N36 and C34_{KQ} (G) are shown. A hydrogen bond is depicted by a blue dashed line. An apostrophe, upper right of an amino acid, means the amino acids of a neighboring helix pair as indicated in (A).

Table 2
Antiviral activity of HIV-1 gp41-derived peptides against recombinant viruses.

Viruses	EC ₅₀ ^a (nM)			
	ddC	DS5000	C34	N36
HIV-1 _{WT}	505 ± 15 ^b	56 ± 8.2	2.8 ± 1.2	200 ± 13
HIV-1 _{D36G}	640 ± 76 ^c (1.3)	94 ± 22 (1.7)	2.8 ± 1.2 (1)	250 ± 46 (1.3)
HIV-1 _{N126K}	515 ± 86 (1)	88 ± 19 (1.5)	11 ± 3.1 (4)	550 ± 105 (2.8)
HIV-1 _{E137Q}	500 ± 90 (1)	84 ± 14 (1.5)	3.0 ± 1.0 (1.1)	636 ± 159 (3.2)
HIV-1 _{N126K/E137Q}	642 ± 84 (1.3)	74 ± 6.0 (1.3)	12 ± 4.7 (4.4)	731 ± 121 (3.7)
HIV-1 _{D36G/N126K/E137Q}	728 ± 29 (1.4)	40 ± 4.0 (0.7)	15 ± 1.0 (5.2)	796 ± 144 (4)
HIV-1 _{gp120-P183Q}	414 ± 50 (0.8)	58 ± 7.5 (1)	2.7 ± 0.6 (1)	343 ± 76 (1.7)
HIV-1 _{gp120-P183Q/D36G/N126K/E137Q}	634 ± 150 (1.2)	43 ± 5.7 (0.8)	15 ± 2.4 (5.2)	573 ± 64 (2.9)

^a Anti-HIV activity was determined with the MAGI assay.

^b The data shown are mean value and standard deviation (SD) obtained from the results of at least three independent experiments.

^c Values in parentheses represent fold-resistance in EC₅₀ for recombinant viruses compared to HIV-1_{WT}.

faster entry kinetics shows resistance to T-20 (Reeves et al., 2002; Heredia et al., 2007; Hermann et al., 2009). However, only CCR5 tropic HIV-1s were subjected to be examined the entry kinetics and T-20 susceptibility in these studies, while CXCR4 tropic HIV-1s have been less examined yet. Interestingly, cell surface expression level of CCR5 influenced T-20 susceptibility but those of CD4 did not (Heredia et al., 2007), suggesting that entry kinetics of HIV-1 only through CCR5 influences T-20 susceptibility. In contrast, our study and others also revealed that D36G, the faster virus with CXCR4 tropism, showed high susceptibility to T-20 (Ueno et al., 2009; Mink et al., 2005; Kinomoto et al., 2005). In the present study, the faster viruses with mutations, such as P183Q and D36G little provided N36 resistance (Table 2). Thus, it is likely that there are some unknown differences in fusion inhibitor susceptibility between CXCR4 and CCR5 tropic viruses. As one of possibilities, Kahle et al. (2009) reported interesting data that asymmetric deactivation is observed in the C-HR targeted 5-helix peptide. Activity of the C-HR-derived peptide, C37 is well-correlated with binding affinity to the N-HR (K_D value), while that of 5-helix targeted the C-HR is poorly-correlated. They proposed a novel mechanism of fusion inhibition that peptides targeting N-HR and C-HR have distinctively reversible and irreversible deactivation of gp41 function, respectively.

D36G is one of the characteristic substitutions or polymorphisms for HIV-1_{NL4-3}, since only HIV-1_{NL4-3} has D36 and others originally contains G36 (Kuiken et al., 2009). Kinomoto et al. (2009) analyzed D36G effect in a structure modeling with SIV gp41 and revealed D36 could induce distortion or incorrect positioning of the N and C helices by misdirected salt bridge with K144. However, less difference between the N36 conformations of wild-type and D36G mutant was expected, since the D36G, the second amino acid from the N-terminal end of N36, is located in the flexible region of N36 sequence. Experiments with the N-HR-derived peptide including expanded N-terminal region of N36 will be needed to reveal structural feature of D36G in the N-HR. In addition to a role in the viral entry, D36G has another role in viral replication kinetics as nucleotides (D_{GAU}36G_{GGU}) in the stem II_C of Rev responsive element (Ueno et al., 2009; Nameki et al., 2005). Nameki et al. (2005) demonstrated that A_{GCC}30V_{GUC} located outside of the N-HR but complementally with D_{GAU}36 in the stem II_C, also enhanced replication kinetics of HIV-1_{NL4-3} (underlined nucleotides; complementally located in the stem II_C) through compensation of the RNA structure. As such, D36G has at least 2 advantages, fusion and RNA stability for viral replication. In the present study, we, therefore, focused mainly on mutations in the C-HR region. In the CD analysis, enhanced 6-helix bundle stability of N36/C34_{KQ} was observed, while structural alteration of the 6-helix bundle was not apparent. We hypothesize that the mechanism of resistance to N36 has little association with the binding ability of C-HR. Although the resistance of HIV-1 to C-HR-derived peptides accounts for a correlation with the binding affinity of N-HR, an important factor

in the resistance to N36 may exist before completion of 6-helix bundle formation. Steger et al. reported that the 5-helix fusion inhibitor, containing three N-HR and two C-HR segments, targeting the C-HR region in gp41 as well as N36, demonstrated poor correlation between inhibitory potency and interaction affinity with C-HR (Steger and Root, 2006). The IC₅₀ values of 5-helix variants with some mutations in the sequence were inversely proportional to their association rate constants. It is consistent with observations by Kahle et al. (2009) mentioned above that mechanism of inhibition of peptides targeting the N- and C-HRs seems to be different, deactivation of gp41 function and dominant negative/decoy effect, respectively. The anti-HIV activity of N36, which has the same target as the 5-helix variants, might be also correlated with association rate constants to C-HR and finally irreversibly deactivate the gp41 function.

In conclusion, non-aggregating and trimeric coiled-coil N-HR-derived peptides such as (CCIZN17)₃ have been developed and exhibit more potent anti-HIV-1 activity (Bianchi et al., 2005; Eckert and Kim, 2001a), as well as exerting a strong synergistic effect with T-20 (Bianchi et al., 2005). Therefore, N-HR-derived peptides with appropriate modifications are promising because of possible co-administration with T-20 and modulation of the resistance profile. The mechanism of resistance to N36, described in this paper, provides a role for the N126K and E137Q mutations in 6-helix bundle stability, although N126 does not directly associate with the N36 surface. Further experiments are needed to clarify the role of the common N126K mutation in HIV-1 fusion.

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Resistance Profiles of Novel Electrostatically Constrained HIV-1 Fusion Inhibitors*

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Human immunodeficiency virus (HIV) gp41 plays a key role in viral fusion; the N- and C-terminal heptad repeats (N-HR and C-HR) of gp41 form a stable 6-helical conformation for fusion. Therefore, HR-derived peptides, such as enfuvirtide (T-20), inhibit HIV-1 fusion by acting as decoys, and have been used for the treatment of HIV-1 infection. However, the efficacy of T-20 is attenuated by resistance mutations in gp41, including V38A and N43D. To suppress the resistant variants, we previously developed electrostatically constrained peptides, SC34 and SC34EK, and showed that both exhibited potent anti-HIV-1 activity against wild-type and T-20-resistant variants. In this study, to clarify the resistance mechanism to this next generation of fusion inhibitors, we selected variants with resistance to SC34 and SC34EK *in vitro*. The resistant variants had multiple mutations in gp41. All of these mutations individually caused less than 6-fold resistance to SC34 and SC34EK, indicating that there is a significant genetic barrier for high-level resistance. Cross-resistance to SC34 and SC34EK was reduced by a simple difference in the polarity of two intramolecular electrostatic pairs. Furthermore, the selected mutations enhanced the physicochemical interactions with N-HR variants and restored activities of the parental peptide, C34, even to resistant variants. These results demonstrate that our approach of designing gp41-binding inhibitors using electrostatic constraints and information derived from resistance studies produces inhibitors with enhanced activity, high genetic barrier, and distinct resistance profile from T-20 and other inhibitors. Hence, this is a promising approach for the design of future generation peptide fusion inhibitors.

The *env* gene of human immunodeficiency virus (HIV) encodes two glycoproteins, gp120 and gp41, that form a stable trimeric complex consisting of three heterodimers to constitute a functional envelope (1). Entry of HIV into target cells is initiated by the interaction of gp120 with the receptor, CD4, and then with co-receptors CCR5 or CXCR4, which are expressed on the target cell surface (2). After receptor binding, a conformational change in gp41 is induced, triggering the exposure of the N-terminal heptad repeat (N-HR)⁴ by stretching the folded gp41, enabling a hydrophobic fusion domain located at the N terminus to be inserted into the target cell membrane (3). Subsequently, the N-HR folds into its counterpart, the C-terminal heptad repeat (C-HR), and together they form a hairpin-like structure of antiparallel helices (6-helix bundle) bringing together and facilitating the fusion of the viral and cellular membranes (4, 5).

Based on the nature of the mechanism of HIV fusion, peptides corresponding to N-HR or C-HR of HIV fusion acted as decoys and interfered with formation of the 6-helix bundle (6, 7). Indeed, a C-HR-derived peptide, enfuvirtide (T-20), suppresses HIV-1 replication, and has been widely used for treatment of HIV-1 infection (8, 9). However, during long-term therapy, T-20-resistant variants emerge among patients treated with T-20-containing regimens (10, 11). To suppress replication of such variants and obtain sustained efficacy, the next generation of fusion inhibitors is urgently needed.

Recently, a number of next generation peptide fusion inhibitors has been reported. These inhibitors include tifuvirtide (T-1249) (12), T-2410 (13), and sifuvirtide (14), which are able to suppress T-20-resistant variants. We have developed electrostatically constrained fusion inhibitors, SC34 and SC34EK, which inhibit replication of T-20-resistant HIV-1 (15). SC34 was designed to be more soluble and have enhanced α -helicity, by engineering electrostatic interactions between glutamic acid and lysine substitutions at *i* and *i*+4 positions in the solvent-interacting site (EK motif) (16) of the parental C-HR-derived C34 peptide (Fig. 1) (17). SC34EK, which has unidirectional EK motifs, demonstrated a 5-fold enhanced activity compared with the original C34 (15, 17). We demonstrated that the α -helical structure was stabilized by electrostatic in-

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⁴ The abbreviations used are: N-HR, N-terminal heptad repeat; C-HR, C-terminal heptad repeat; MAGI, multinuclear activation of a galactosidase indicator.

Resistance Profile of SC34 and SC34EK

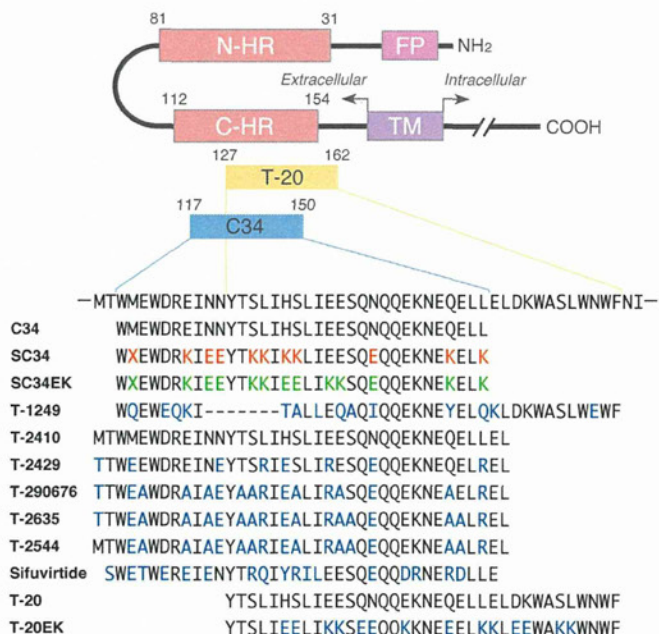


FIGURE 1. Peptide sequences of gp41-derived fusion inhibitors. C-HR-derived fusion inhibitors are shown with the functional domains of HIV-1 gp41. The residue numbers of each peptide correspond to their positions in gp41 of HIV-1_{NL4-3}. FP, fusion peptide; TM, transmembrane domain; X, norleucine (Nle); —, deleted amino acid.

teractions of the EK motif with specific residues on the target peptide, providing high selectivity (15).

In addition to engineering stabilizing electrostatic interactions we have also established a second strategy to design improved peptide fusion inhibitors. This strategy is based on the introduction of resistance mutations into the sequence of the original peptide inhibitor. These new changes enhance the antiviral potency against resistant variants. Hence, T-20 with a S138A substitution (T-20_{S138A}), one of the secondary resistance mutations observed in patients that fail to respond to T-20, regained anti-HIV-1 activity against T-20-resistant variants (18). X-ray crystallographic and circular dichroism (CD) analyses revealed that the S138A substitution contributed to stability of the N-HR-C-HR complex (19). Similar results were observed for C34 with N126K (C34_{N126K}), also secondary mutations for T-20-resistant variants *in vivo* (20, 21) and C34-resistant variants *in vitro* (18). Therefore, the novel strategy to design inhibitor peptides utilizing resistance mutations has resulted in antivirals that can suppress variants resistant to the parental peptides.

In this study, to determine the mechanism of resistance and the effect of escape mutations on the potency of the next generation fusion inhibitors, we induced resistant variants to SC34 and SC34EK *in vitro*. Our results demonstrate that peptides that are designed to have specific electrostatic constraints and include changes that are based on resistance information have significantly improved properties in terms of potency and cross-resistance.

EXPERIMENTAL PROCEDURES

Antiviral Agents—Peptide fusion inhibitors (Fig. 1) were synthesized based on a previous report (17). 2',3'-Dideoxycytidine was purchased from Sigma.

Cells and Viruses—MT-2 and 293T cells were grown in RPMI1640 medium and Dulbecco's modified Eagle's medium (DMEM), respectively. These media were supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml of penicillin, and 50 μg/ml of streptomycin. HeLa-CD4/CCR5-LTR/β-gal cells (22) (provided by Dr. J. Overbaugh through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID) were maintained in DMEM supplemented with 10% FCS, 200 μg/ml of hygromycin B, 10 μg/ml of puromycin, and 200 μg/ml of geneticin.

An HIV-1 molecular clone, pNL4-3 (23), was employed as a wild-type HIV-1 (HIV-1_{NL4-3}), and used for the construction of various gp41-recombinant viruses as described previously (15, 18, 24, 25). The viruses were harvested from the supernatant of transfected 293T cells and stored at -80 °C.

Determination of Drug Susceptibility—Anti-HIV activity of inhibitors was determined using multinuclear activation of a galactosidase indicator (MAGI) assay as described previously (15, 18, 24–26). Inhibitory activity was presented as the concentration required for 50% inhibition (EC₅₀).

Dose Escalating Induction of Resistant Variants to SC34 or SC34EK—MT-2 cells were initially infected with HIV-1_{NL4-3} in the presence of 0.1 nM SC34 or 0.15 nM SC34EK. Infected cells were incubated at 37 °C until a cytopathic effect was observed. When an extensive cytopathic effect was observed, culture supernatant was harvested and used for the next round of infection on fresh MT-2 cells in the presence of a 2-fold increased concentration of inhibitors as shown in Fig. 2. At the indicated passages, proviral DNA was extracted from infected cells and the mutations were identified by direct sequencing. These selections with dose escalations were performed in a single HIV-1 selection culture. To avoid selection of a minor population for drug resistance, in each passage, we propagated the virus after an extensive cytopathic effect was observed.

Replication Kinetics of Env-recombinant Viruses—MT-2 cells (10⁵ cells/3 ml) were infected with each env-recombinant HIV-1 clone (500 MAGI units) for 4 h. The infected cells were extensively washed and cultured in 4 ml in 6-well plates. The culture supernatants were harvested periodically and production of progeny viruses was monitored by the MAGI assay.

Circular Dichroism (CD) Spectroscopic Analysis—CD analysis was performed as described previously (15, 18, 27), with some modifications. In brief, spectra of a complex of N-HR and C-HR peptides (each 10 μM) or an N-HR peptide alone (10 μM) in 5 mM HEPES buffer (pH 7.2) were collected using a J-710 CD spectrometer (Jasco, Tokyo, Japan) equipped with a thermoelectric temperature controller. The thermal stability was measured as the change of CD signal at 222 nm. The temperature that resulted in 50% unfolding (melting temperature, T_m) of each complex was determined.

Statistical Analysis—Pearson correlation analysis of relationships between T_m values of each N-HR-C-HR complex determined in the CD analysis, and the EC₅₀ values of inhibitors determined by MAGI assay, was performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA). *p* values less than 0.05 were considered statistically significant.

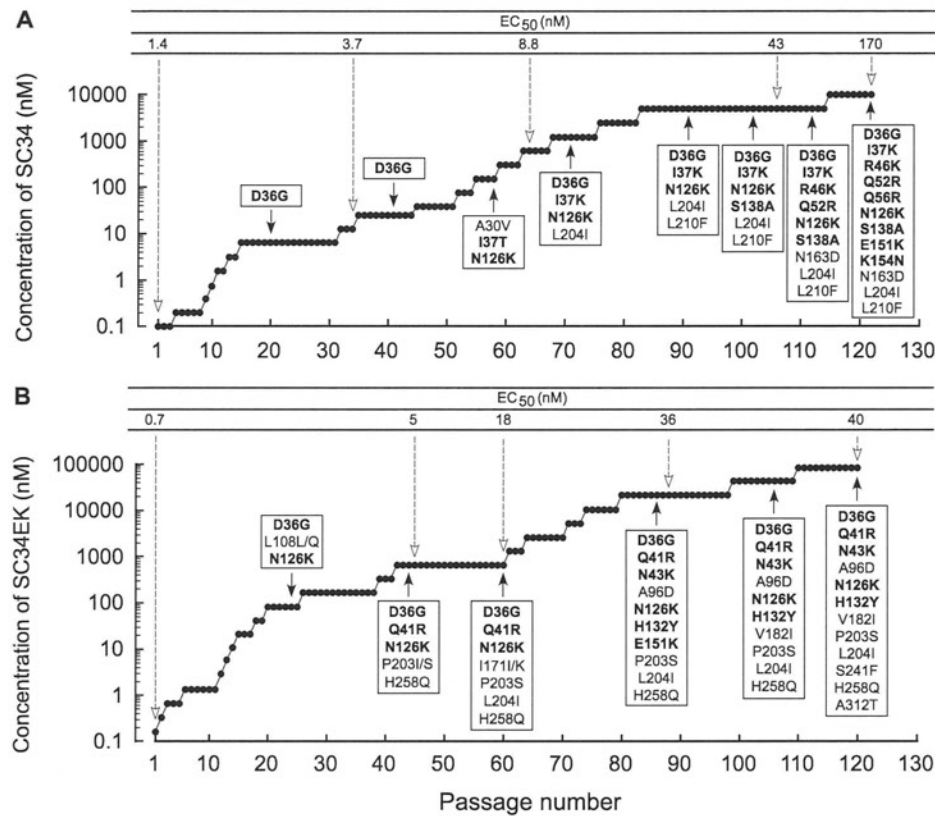


FIGURE 2. Induction of SC34- and SC34EK-resistant HIV-1. HIV-1_{NL4-3} was passaged in the presence of increasing concentrations of the inhibitors in MT-2 cells. The dose escalating selections were carried out for a total of 122 passages with SC34 concentrations ranging from 0.1 nM to 9.8 μ M (A), and for 120 passages with SC34EK concentrations ranging from 0.15 nM to 80 μ M (B). At the indicated passage number (black arrowheads with solid line), proviral DNA was extracted from infected cells, and gp41- and gp120-coding regions were sequenced. At the final passage (P-122 for SC34 and P-120 for SC34EK), the following gp120 mutations were identified; K107K/Q (mixture of K and Q), S134N, S136G, and F147L in the case of SC34, and V37A, V59I, S100K, S115N, R138S, D139N, and A310T in the case of SC34EK. Amino acids shown in bold are located within the N-HR (31–81) or C-HR (112–154) in the gp41. The EC₅₀ values of HIV-1 variants (heterogeneous pool) at the indicated passage number (white arrowheads with dashed line) were determined using the MAGI assay.

RESULTS

Selection of SC34-resistant HIV-1 in Vitro—To determine the resistance profile of SC34, SC34-resistant variants were selected by a dose-escalating method and susceptibility of the obtained variants was determined by the MAGI assay. Selection of resistant HIV-1_{NL4-3} was initially started in the presence of 0.1 nM SC34 (Fig. 2A). At passage 20 (P-20), where the concentration of SC34 was 6.4 nM, substitution of aspartic acid to glycine at position 36 in the gp41 coding region (D36G) was observed. At P-58, although the D36G substitution transiently reverted to the original amino acid, a set of mutations, A30V, I37T, and N126K, which also emerged during the induction of resistance to SC34 (24), was introduced. At P-71, D36G was again observed, and simultaneously, L204I was introduced, whereas A30V disappeared. L210F and S138A were newly introduced at P-91 and P-102, respectively. The EC₅₀ of SC34 to the P-106 variant increased to 43 nM. At P-112, further substitutions, R46K, Q52R, and N163D, were added. The amino acid at position 163 was predominantly aspartic acid among many HIV-1 strains (28). Therefore, the N163D change was considered to be a polymorphism. At the final passage, P-122, Q56R, E151K, and K154N were further introduced, and the EC₅₀ for SC34 reached 170 nM. In addition to the mutations introduced in the gp41, some mutations

were also identified in gp120 at P-122 (K107K/Q (a mixture of Lys and Gln), S134N, S136G, and F147L).

Selection of SC34EK-resistant HIV-1 in Vitro—Induction of SC34EK resistance was also performed, except that the initial concentration of SC34EK was 0.15 nM (Fig. 2B). At P-24, two substitutions, D36G and N126K, were observed in the gp41. A transient partial substitution at Leu¹⁰⁸ was observed as a mixture of leucine and glutamine (L108L/Q), but disappeared at later passages. At P-44, Q41R, P203I/S, and H258Q were added. Heterogeneous P203I/S at P-44 was transient, and was predominantly substituted by P203S at P-60. The EC₅₀ of SC34EK to P-45 variants was 5 nM. At P-60, I171I/K and L204I were added, and the EC₅₀ increased to 18 nM. At P-86, four further substitutions, N43K, A96D, H132Y, and E151K, were newly introduced. The EC₅₀ of SC34EK to the P-88 variant increased to 36 nM. Further substitutions, V182I and S241F/A312T, emerged at P-106 and P-120, respectively. The EC₅₀ of SC34EK for the P-120 variant ultimately reached 40 nM. H132Y and V182I observed at later passages were considered gp41 polymorphisms, because these were predominantly observed in non-treated clinical isolates (28). Mutations introduced in the gp120 at the final P-120 were V37A, V59I, S100K, S115N, R138S, D139N, and A310T.

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TABLE 1

Susceptibility of SC34-selected mutation-introduced env-recombinant viruses to fusion inhibitors

Anti-HIV activity was determined using MAGI assay. Data are shown as mean \pm S.D. obtained from at least three independent experiments, and resistance (*n*-fold of the EC₅₀) of recombinant viruses, compared to that of parental HIV-1_{NL4-3}^a, is shown in parentheses.

Mutation(s)	EC ₅₀ (nM)				
	ddC	T-20	C34	SC34	SC34EK
HIV-1 _{NL4-3} ^a	430 \pm 121	20 \pm 3	3.3 \pm 0.6	1.4 \pm 0.3	0.7 \pm 0.4
gp41					
A30V	213 \pm 40 (0.5)	6.4 \pm 2.2 (0.3)	7.2 \pm 1.6 (2.2)	0.5 \pm 0.2 (0.4)	0.8 \pm 0.2 (1.1)
D36G	392 \pm 93 (0.9)	1.0 \pm 0.1 (0.05)	8.1 \pm 3.4 (2.5)	0.6 \pm 0.2 (0.4)	0.7 \pm 0.2 (1.0)
I37K	287 \pm 92 (0.7)	2,453 \pm 873 (123)	23 \pm 3 (7.0)	4.9 \pm 0.6 (3.5)	1.0 \pm 0.2 (1.4)
I37T	289 \pm 30 (0.7)	166 \pm 40 (8.3)	20 \pm 5 (6.1)	2.3 \pm 0.8 (1.6)	0.5 \pm 0.1 (0.7)
R46K	572 \pm 162 (1.3)	ND ^b	1.3 \pm 0.4 (0.4)	0.6 \pm 0.1 (0.4)	1.9 \pm 0.2 (2.7)
Q52R	415 \pm 29 (1.0)	ND	2.9 \pm 0.8 (0.9)	1.6 \pm 0.5 (1.1)	1.5 \pm 0.2 (2.1)
Q56R	300 \pm 58 (0.7)	ND	28 \pm 6 (8.5)	4.1 \pm 1.0 (2.9)	1.0 \pm 0.2 (1.4)
N126K	256 \pm 18 (0.6)	27 \pm 5 (1.4)	8.0 \pm 2.9 (2.4)	1.3 \pm 0.4 (0.9)	0.4 \pm 0.1 (0.6)
S138A	210 \pm 64 (0.5)	ND	4.0 \pm 0.8 (1.2)	2.3 \pm 0.8 (1.6)	0.8 \pm 0.2 (1.1)
E151K	277 \pm 16 (0.6)	ND	2.3 \pm 0.6 (0.7)	1.1 \pm 0.02 (0.8)	1.4 \pm 0.4 (2.0)
K154N	529 \pm 160 (1.2)	ND	2.1 \pm 1.7 (0.6)	1.4 \pm 0.5 (1.0)	0.4 \pm 0.2 (0.6)
N163D	348 \pm 33 (0.8)	ND	0.5 \pm 0.1 (0.2)	2.5 \pm 0.8 (1.8)	0.9 \pm 0.7 (1.3)
L204I	226 \pm 12 (0.5)	13 \pm 4 (0.7)	3.9 \pm 0.1 (1.2)	1.5 \pm 0.3 (1.1)	0.6 \pm 0.1 (0.9)
L210F	556 \pm 162 (1.3)	ND	4.1 \pm 1.7 (1.2)	0.6 \pm 0.1 (0.4)	0.4 \pm 0.04 (0.6)
SC34(P-122)gp41 ^c	329 \pm 33 (0.8)	1,727 \pm 255 (86)	24 \pm 4 (7.3)	2,189 \pm 287 (1,564)	23 \pm 1 (33)
gp120					
SC34(P-122)gp120 ^d	339 \pm 95 (0.8)	45 \pm 2 (2.3)	0.8 \pm 0.3 (0.2)	0.2 \pm 0.03 (0.1)	0.2 \pm 0.05 (0.3)
gp160					
SC34(P122)gp160 ^e	542 \pm 68 (1.3)	>5,000 (>250)	>1,000 (>303)	2,827 \pm 439 (2,019)	33 \pm 4 (47)

^a HIV-1_{NL4-3} was used as wild-type virus.

^b ND, not determined.

^c HIV-1_{SC34(P-122)gp41} contains D36G/I37K/R46K/Q52R/Q56R/N126K/S138A/E151K/K154N/N163D/L204I/L210F mutations in gp41 coding region.

^d HIV-1_{SC34(P-122)gp120} contains K107Q/S134N/S136G/F147L mutations in gp120 coding region.

^e HIV-1_{SC34(P-122)gp160} contains K107Q/S134N/S136G/F147L and D36G/I37K/R46K/Q52R/Q56R/N126K/S138A/E151K/K154N/N163D/L204I/L210F mutations in gp120 and gp41 coding regions, respectively.

Acquisition of resistance to SC34EK seems to be faster than that to SC34 (Fig. 2). However, actual EC₅₀ values for SC34 and SC34EK remain just 8.5- and 2-fold of that of T-20, respectively.

Phenotypic Analysis of gp41-, gp120-, and gp160-recombinant HIV-1—To determine which mutations contributed to inhibitor resistance we constructed gp41 recombinant HIV-1s containing each of the mutations that emerged during resistant variant selection. Changes of susceptibility were determined by the MAGI assay (Tables 1 and 2). During T-20 therapy, HIV-1 acquired T-20 resistance mutations, especially in amino acids at positions 36–45 (DIVQQNNLL in the NL4–3 sequence) of gp41 N-HR, where it interacts with C-HR, mainly contributing to the resistance (29–32). In our selections using SC34 and SC34EK, several mutations were also located within this region, and some conferred T-20 resistance to various extents (123- and 14-fold by I37K and N43K, respectively). I37K was slightly resistant to SC34 (3.5-fold). Ile³⁷ was initially substituted with threonine (I37T) at P-58, and later converted to lysine (I37K) at P-71, moderately reducing the susceptibility to SC34 (from 1.6- to 3.5-fold). Other substitutions had little influence on the susceptibility to SC34 (0.4–2.9-fold decreases). However, HIV-1_{SC34(P-122)gp41}, which contains multiple substitutions, D36G/I37K/R46K/Q52R/Q56R/N126K/S138A/E151K/K154N/N163D/L204I/L210F, showed high resistance to SC34 (1564-fold) and T-20 (86-fold), but mild and moderate resistance to C34 (7.3-fold) and SC34EK (33-fold), respectively.

The A96D mutation observed in SC34EK selection conferred high resistance to T-20 (more than 50-fold), but only weak resistance to SC34EK (6.3-fold) (Table 2). Ala⁹⁶ is located between two HRs, termed a loop or hinge region, and so

far, there are no clinical reports that A96D is involved in T-20 resistance during antiviral therapy. It is likely that the A96D substitution that introduces a larger polar residue at position 96 induces structural changes to gp41, resulting in reduced T-20 interaction. However, the exact effects of the A96D mutation on the resistance remain unclear. An N43K mutation, which is observed in T-20-treated HIV-1-infected patients, decreased susceptibility to C34 (55-fold), whereas it had little effect on the susceptibility to SC34 (1.9-fold) and SC34EK (3.3-fold). An H132Y substitution also conferred mild and moderate resistance to SC34EK and T-20 (4.9- and 18-fold, respectively), but not to C34 and SC34. Substitutions other than A96D and H132Y had little influence on the susceptibility to SC34EK (range of fold-decrease in resistance was 0.6–3.3). However, HIV-1_{SC34EK(P-120)gp41}, which contains multiple substitutions, D36G/Q41R/N43K/A96D/N126K/H132Y/V182I/P203S/L204I/S241F/H258Q/A312T, again conferred high resistance to SC34EK (107-fold), but moderate resistance to both C34 (21-fold) and SC34 (14-fold).

The vast majority of HIV-1 strains carry a glycine at position 36 (G36), but in rare cases, NL4-3 harbors aspartic acid (Asp³⁶) (28). We recently observed that D36G is selected during passages even in the absence of peptide inhibitors,⁵ indicating that D36G is an HIV-1_{NL4-3}-specific substitution.

We also evaluated the effect of mutations in the gp120 on drug susceptibility. The two clones, HIV-1_{SC34(P-122)gp120} and HIV-1_{SC34EK(P-120)gp120} containing K107Q/S134N/S136G/F147L and V37A/V59I/S100K/S115N/R138S/D139N/A310T mutations, respectively, showed no significant resistance to

⁵ K. Shimura, D. Nameki, K. Kajiwara, K. Watanabe, Y. Sakagami, S. Oishi, N. Fujii, M. Matsuoka, S. G. Sarafianos, and E. Kodama, unpublished data.

TABLE 2

Susceptibility of SC34EK-selected mutation-introduced env-recombinant viruses to fusion inhibitors

Anti-HIV activity was determined using MAGI assay. Data are shown as mean \pm S.D. obtained from at least three independent experiments, and resistance (n -fold of the EC_{50}) of recombinant viruses, compared to that of parental HIV-1_{NL4-3}, is shown in parentheses.

Mutation(s)	EC_{50} (nM)				
	ddC	T-20	C34	SC34	SC34EK
HIV-1 _{NL4-3} ^a	430 \pm 121	20 \pm 3	3.3 \pm 0.6	1.4 \pm 0.3	0.7 \pm 0.4
gp41					
D36G	392 \pm 93 (0.9)	1.0 \pm 0.1 (0.05)	8.1 \pm 3.4 (2.5)	0.6 \pm 0.2 (0.4)	0.7 \pm 0.2 (1.0)
Q41R	259 \pm 19 (0.6)	ND ^b	125 \pm 50 (38)	5.3 \pm 0.4 (3.8)	2.3 \pm 0.6 (3.3)
N43K	220 \pm 5.0 (0.5)	278 \pm 24 (14)	180 \pm 54 (55)	2.7 \pm 0.6 (1.9)	2.3 \pm 0.8 (3.3)
A96D	255 \pm 13 (0.6)	>1,000 (>50)	12 \pm 1 (3.6)	2.8 \pm 1.2 (2.0)	4.4 \pm 0.9 (6.3)
N126K	256 \pm 18 (0.6)	27 \pm 5 (1.4)	8.0 \pm 2.9 (2.4)	1.3 \pm 0.4 (0.9)	0.4 \pm 0.1 (0.6)
H132Y	394 \pm 83 (0.9)	363 \pm 70 (18)	3.6 \pm 1.4 (1.1)	2.7 \pm 1.3 (1.9)	3.4 \pm 0.4 (4.9)
E151K	277 \pm 16 (0.6)	ND	2.3 \pm 0.6 (0.7)	1.1 \pm 0.02 (0.8)	1.4 \pm 0.4 (2.0)
V182I	686 \pm 181 (1.6)	26 \pm 8 (1.3)	4.5 \pm 1.2 (1.4)	1.3 \pm 0.3 (0.9)	0.7 \pm 0.2 (1.0)
P203S	ND	ND	ND	ND	ND
L204I	226 \pm 12 (0.5)	13 \pm 4 (0.7)	3.9 \pm 0.1 (1.2)	1.5 \pm 0.3 (1.1)	0.6 \pm 0.1 (0.9)
P203S/L204I	646 \pm 208 (1.5)	23 \pm 5 (1.2)	3.5 \pm 0.1 (1.1)	1.6 \pm 0.2 (1.1)	1.5 \pm 0.2 (2.1)
S241F	420 \pm 75 (1.0)	20 \pm 3 (1.0)	6.3 \pm 0.2 (1.9)	1.6 \pm 0.3 (1.1)	0.6 \pm 0.1 (0.9)
H258Q	381 \pm 25 (0.9)	54 \pm 19 (2.7)	13 \pm 3 (3.9)	1.8 \pm 0.4 (1.3)	0.7 \pm 0.4 (1.0)
A312T	ND	ND	ND	ND	ND
H258Q/A312T	633 \pm 140 (1.5)	28 \pm 5 (1.4)	6.0 \pm 0.7 (1.8)	1.3 \pm 0.2 (0.9)	0.7 \pm 0.2 (1.0)
SC34EK(P-120)gp41 ^c	536 \pm 20 (1.2)	112 \pm 38 (5.6)	70 \pm 9 (21)	20 \pm 1 (14)	75 \pm 8 (107)
gp120					
SC34EK(P-120)gp120 ^d	399 \pm 85 (0.9)	70 \pm 16 (3.5)	1.4 \pm 0.4 (0.4)	0.2 \pm 0.03 (0.1)	0.3 \pm 0.1 (0.4)
gp160					
SC34EK(P-120)gp160 ^e	344 \pm 42 (0.8)	435 \pm 139 (22)	378 \pm 133 (115)	5.2 \pm 0.5 (3.7)	72 \pm 18 (103)

^a HIV-1_{NL4-3} was used as wild-type virus.

^b ND, not determined.

^c HIV-1_{SC34EK(P-120)gp41} contains D36G/Q41R/N43K/A96D/N126K/H132Y/V182I/P203S/L204I/S241F/H258Q/A312T mutations in gp41-coding region.

^d HIV-1_{SC34EK(P-120)gp120} contains V37A/V59I/S100K/S115N/R138S/D139N/A310T mutations in gp120-coding region.

^e HIV-1_{SC34EK(P-120)gp160} contains V37A/V59I/S100K/S115N/R138S/D139N/A310T and D36G/Q41R/N43K/A96D/N126K/H132Y/V182I/P203S/L204I/S241F/H258Q/A312T mutations in gp120- and gp41-coding regions, respectively.

T-20, C34, SC34, and SC34EK. Instead, they exhibited hypersensitivity to SC34, SC34EK, and C34, and only moderate resistance to T-20 (less than 4-fold). Moreover, the gp120 mutations barely enhanced resistance conferred by the mutations gp41 (1.3- and 1.0-fold resistance to SC34 and SC34EK, respectively). Of note, when the SC34-selected mutations in gp41 and gp120 were both present in gp160, we observed significant increases in resistance to T-20 (from 86- to >250-fold) and C34 (from 7.3- to >303-fold). Similarly, when the SC34EK-selected mutations in gp41 and gp120 were present at the same time, we observed an increase in resistance to T-20 from 5.6- to 22-fold and to C34 from 21- to 115-fold. These results indicate that resistance to SC34 and SC34EK require accumulation of multiple mutations, rather than certain primary mutation(s) in the gp41, which is distinct from the mechanism of resistance to T-20 and C34.

Replication Kinetics of Env-recombinant HIV-1—The effects of SC34- and SC34EK-selected mutations in gp120 and gp41 on HIV-1 replication kinetics were assessed by measuring production levels of infectious virions in the culture supernatant by MAGI assay. Replication kinetics of clones containing SC34-selected mutations were little and moderately reduced by mutations in gp120 and gp41, respectively (Fig. 3). The reduced replication capacity observed in HIV-1_{SC34(P-122)gp41} was partially restored by mutations in the gp120 (HIV-1_{SC34(P-122)gp160}). Similarly, SC34EK-selected mutations in gp120 partially improved the replication capacity of HIV-1_{SC34EK(P-120)gp41}, which was severely impaired by mutations in gp41. Taken together, these results and phenotypic analyses (Tables 1 and 2) indicate that SC34- and SC34EK-selected mutations in the gp41, not in the gp120, are

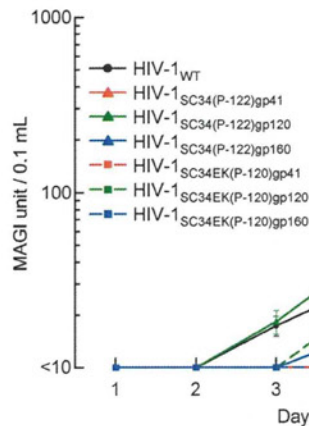


FIGURE 3. Replication kinetics of env-recombinant HIV-1 variants. The effects of SC34- and SC34EK-selected mutations on the replication kinetics were analyzed. MT-2 cells were infected with each env-recombinant HIV-1 variant at 500 MAGI units. After a 4-h incubation, the infected cells were washed and cultured for 7 days. The culture supernatants were harvested every 24 h and production of progeny viruses was monitored by MAGI assay. HIV-1_{NL4-3} was used as wild-type strain (HIV-1_{WT}). The presented data are mean \pm S.D. of MAGI units obtained from 0.1 ml of culture supernatants. Results shown are representative of three independent experiments each using two independent clones. Error bars on each point represent the S.D. of the mean.

mainly involved in resistance to SC34 and SC34EK with replication cost, whereas mutations in the gp120 partially restore the replication capacity through secondary mutations.

Effect of Mutations on the Stability of the α -Helical Bundle Formation—To elucidate the impact of SC34- and SC34EK-selected mutations introduced in N-HR and C-HR on the stability of complex formation between N-HR and C-HR, CD analysis was performed. The N36 peptide was used as a wild-

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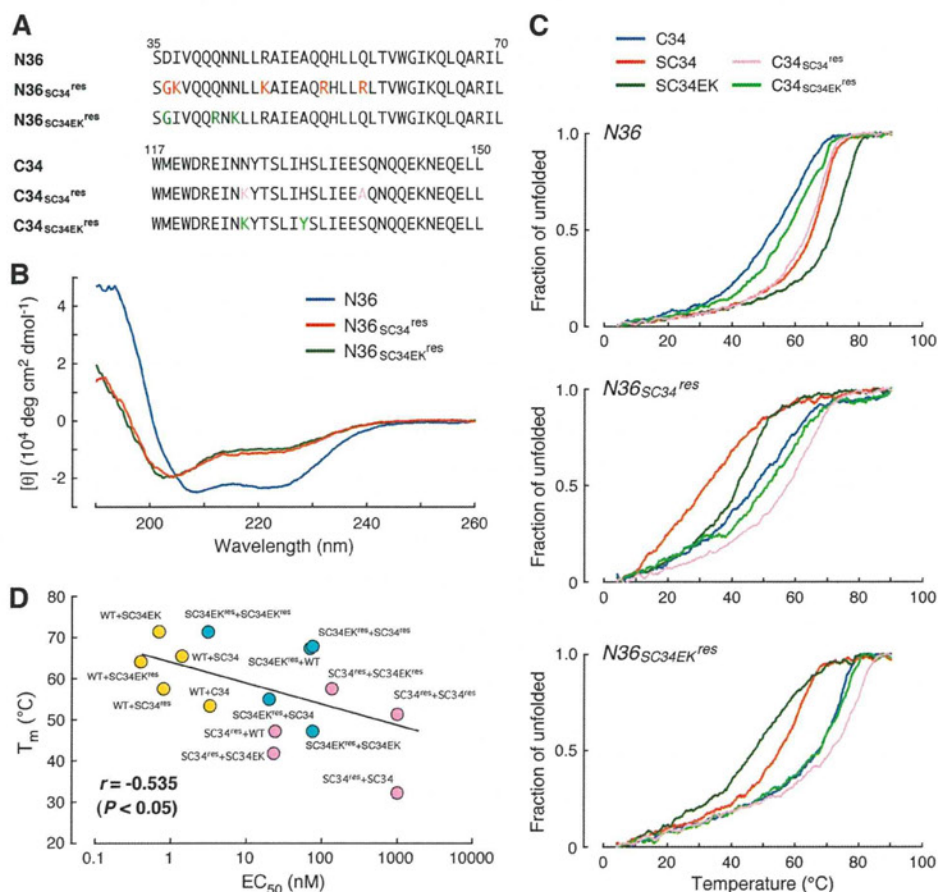


FIGURE 4. CD analysis of physicochemical properties of fusion inhibitors. *A*, modified peptides with mutations selected by SC34 or SC34EK were chemically synthesized. The mutations observed in the N-HR and C-HR were introduced into the original sequences of N36 and C34, respectively. Substituted amino acids are shown in color. *B*, CD spectra change of N-HR peptide alone. Original N36, SC34-selected mutation-introduced N36 (N36_{SC34}^{res}), and SC34EK-selected mutation-introduced N36 (N36_{SC34EK}^{res}) were separately prepared and their α -helicities, defined as molar ellipticity (θ), were examined. *C*, thermal midpoint analysis was measured at 222 nm for N-HR (N36) and C-HR (C34) peptide complexes. Equimolar amounts of N-HR and C-HR were mixed and the thermal-induced unfolding transition was measured. Original N36 (top), N36_{SC34}^{res} (middle), and N36_{SC34EK}^{res} (bottom) were used as N-HRs. C34, SC34, SC34EK, and mutation-introduced C34 (C34_{SC34}^{res} and C34_{SC34EK}^{res}) are indicated in color. *D*, correlation between T_m values of each N-HR-C-HR complex determined in *C* and antiviral activity of C-HR peptides (EC_{50} values determined by MAGI assay). Combination of N-HR + C-HR peptide pairs is indicated as "N-HR + C-HR" in each plot. Orange, original N36; pink, N36_{SC34}^{res}; blue, N36_{SC34EK}^{res}. Pearson correlation coefficient (r) and its p value were calculated.

type N-HR. The mutant peptides, N36_{SC34}^{res} and N36_{SC34EK}^{res}, harboring SC34-selected mutations (D36G/I37K/R46K/Q52R/Q56R) and SC34EK-selected mutations (D36G/Q41R/N43K) were prepared through chemical synthesis (Fig. 4A). C34_{SC34}^{res} and C34_{SC34EK}^{res} that contain N126K/S138A and N126K/H132Y substitutions, respectively, were also synthesized.

First, we compared the α -helicity of each N-HR peptide alone. In contrast to native N36, no typical features of α -helicities were observed in either N36_{SC34}^{res} or N36_{SC34EK}^{res} (Fig. 4B). Additionally, few significant temperature-dependent changes of spectra were observed in both N36_{SC34}^{res} and N36_{SC34EK}^{res} over a broad range of temperatures compared with N36 (data not shown). This indicated that both inhibitors resulted in the selection of N-HR mutations that decreased the helical content and only the original N36 folded into an intramolecular coiled-coil form and behaved as an α -helix.

Thermal stability of the complex formed between N-HR and C-HR was investigated. The midpoint of the thermal un-

folding transition (T_m) of N36-C34 was 53.3 °C, whereas N36-SC34 and N36-SC34EK formed more stable complexes (T_m values of 65.4 °C for N36-SC34 and 71.4 °C for N36-SC34EK; Fig. 4C). The resistance mutations in the C-HR enhanced binding stability; T_m of 64.1 °C for N36-C34_{SC34}^{res} and 57.5 °C for N36-C34_{SC34EK}^{res}. C34, C34_{SC34}^{res}, and C34_{SC34EK}^{res} maintained binding capacity to both N36_{SC34}^{res} and N36_{SC34EK}^{res} (Fig. 4C). SC34 and SC34EK no longer formed stable complexes with N36_{SC34}^{res} and N36_{SC34EK}^{res}, as their T_m values were lower than C34. These results indicate that substitutions of compensatory mutations into C-HR contribute to stability enhancement. To further evaluate whether decreased susceptibility of the resistant variants to the inhibitors was caused by thermodynamic instability of the peptide bundle formation, we analyzed the relationship between the values of T_m and EC_{50} . The T_m was determined by CD analysis and found to be inversely correlated with the EC_{50} value measured in the MAGI assay (Pearson correlation analysis, $r = -0.535$, $p < 0.05$; Fig. 4D). Overall, these experiments demonstrate that mutations within N-HR reduce its α -helic-

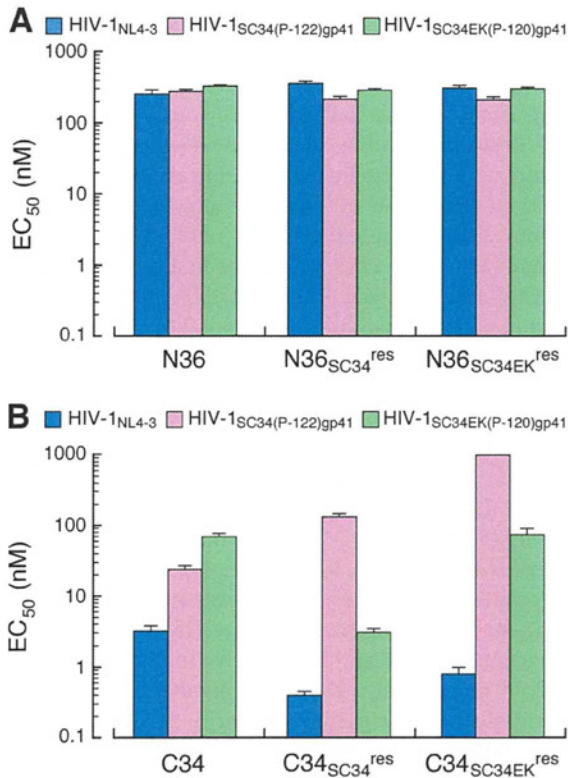


FIGURE 5. Antiviral activity of resistant mutation-introduced peptides. Antiviral activity of resistant mutation-introduced N36 ($N36_{SC34}^{res}$, $N36_{SC34EK}^{res}$, and original N36) (A) or C34 ($C34_{SC34}^{res}$, $C34_{SC34EK}^{res}$, and original C34) peptides (B) against HIV-1_{NL4-3} (blue), HIV-1_{SC34(P-122)gp41} (pink), and HIV-1_{SC34EK(P-120)gp41} (green) was determined using the MAGI assay. Peptide sequences are shown in Fig. 4A. HIV-1_{NL4-3} was used as the wild-type virus. HIV-1_{SC34(P-122)gp41} contained D36G/I37K/R46K/Q52R/Q56R/N126K/S138A/E151K/K154N/N163D/L204I/L210F mutations. HIV-1_{SC34EK(P-120)gp41} had D36G/Q41R/N43K/A96D/N126K/H132Y/V182I/P203S/L204I/S241F/H258Q/A312T mutations. Data are shown as mean EC_{50} values with error bars indicating standard deviations, obtained from at least three independent experiments.

ity and result in thermal instability of the 6-helix bundle by destabilizing the N-HR-C-HR complex. In contrast, mutations in C-HR simply enhanced the binding affinity to both wild-type and mutated N-HR.

Inhibitory Activity of Modified Fusion Inhibitors—We have previously demonstrated that introduction of resistance mutations to T-20 restores anti-HIV-1 activity against T-20-resistant variants (18), suggesting that this strategy can result in the design of peptides with improved potential for the treatment of resistant HIV-1. We examined whether this strategy was also applicable to SC34- and SC34EK-selected mutations. Hence, we introduced SC34- and SC34EK-resistant mutations that emerged in N-HR or C-HR, into the parental N36 or C34 peptides, respectively (Fig. 4A). Anti-HIV activities of both $N36_{SC34}^{res}$ and $N36_{SC34EK}^{res}$ showed little change compared with N36 (Fig. 5A), consistent with the CD analyses shown in Fig. 4, B and C, whereas mutations introduced into C34 restored their activity (Fig. 5B). $C34_{SC34}^{res}$ showed potent activity against HIV-1_{NL4-3} and surprisingly, it also exhibited enhanced activity against HIV-1_{SC34EK(P-120)gp41}. $C34_{SC34EK}^{res}$ moderately suppressed HIV-1_{SC34EK(P-120)gp41}, but was more effective in suppressing HIV-1_{NL4-3}, as compared with C34.

TABLE 3

Antiviral activity of fusion inhibitors to SC34- and SC34EK-selected mutation introduced HIV-1 gp41 recombinant viruses

Anti-HIV activity was determined using MAGI assay. Data are shown as mean \pm S.D. obtained from at least three independent experiments, and resistance (n -fold of EC_{50}) of recombinant viruses, compared to that of parental HIV-1_{NL4-3}, is shown in parentheses.

Inhibitors	EC_{50} (nM)		
	HIV-1 _{NL4-3} ^a	HIV-1 _{SC34(P-122)gp41} ^b	HIV-1 _{SC34EK(P-120)gp41} ^c
T-1249	0.4 \pm 0.07	0.5 \pm 0.06 (1.3)	0.5 \pm 0.03 (1.3)
T-2410	1.0 \pm 0.3	38 \pm 13 (38)	3.7 \pm 0.6 (3.7)
T-2429	2.2 \pm 0.8	5.9 \pm 1.3 (2.7)	5.4 \pm 1.1 (2.5)
T-2544	0.9 \pm 0.3	1.5 \pm 0.5 (1.7)	1.4 \pm 0.5 (1.6)
T-2635	0.3 \pm 0.08	0.4 \pm 0.1 (1.3)	1.1 \pm 0.2 (3.7)
T-290676	0.7 \pm 0.2	2.9 \pm 0.8 (4.1)	4.6 \pm 0.6 (6.6)
Sifuvirtide	1.7 \pm 0.5	340 \pm 55 (200)	35 \pm 8.3 (21)
T-20EK	6.4 \pm 0.8	1,548 \pm 90 (242)	2,650 \pm 261 (414)

^a HIV-1_{NL4-3} was used as wild-type virus.

^b HIV-1_{SC34(P-122)gp41} has D36G/I37K/R46K/Q52R/Q56R/N126K/S138A/E151K/K154N/N163D/L204I/L210F mutations.

^c HIV-1_{SC34EK(P-120)gp41} has D36G/Q41R/N43K/A96D/N126K/H132Y/V182I/P203S/L204I/S241F/H258Q/A312T mutations.

These results further validate our strategy to overcome resistance to peptide fusion inhibitors by incorporating resistance mutations into the sequence of the original peptide inhibitor. Hence, we have been able to design peptides that can overcome resistance to T-20, C34, and now SC34 and SC34EK.

Inhibition of Resistant HIV-1 by Peptide Fusion Inhibitors—Recently, several novel peptides, including SC34 and SC34EK, have been developed as the next generation fusion inhibitors. To compare their antiviral properties, we evaluated their activities against SC34- and SC34EK-resistant HIV-1. All tested peptide fusion inhibitors showed remarkable anti-HIV-1 activity against HIV-1_{NL4-3} with EC_{50} values in the subnanomolar to nanomolar range (Table 3). However, the inhibitors had different effects on SC34- and SC34EK-resistant variants. T-1249, T-2429, T-2544, T-2635, and T-290676 retained activity against HIV-1_{SC34(P-122)gp41}, whereas T-2410, sifuvirtide, and T-20EK showed a decreased effect to various extents. Similarly, sifuvirtide and T-20EK had reduced activity against HIV-1_{SC34EK(P-120)gp41}. These results indicate that only minimal cross-resistance to the next generation of fusion inhibitors might emerge, and suggest possible successful combinations of fusion inhibitors.

DISCUSSION

To date, it remains unclear how the electrostatic constraints that are imposed on a peptide by the incorporation of EK motifs also affect the resistance profile and other virological features of these peptide fusion inhibitors. In this study, we selected HIV variants to the SC34 and SC34EK peptide inhibitors that have EK motifs, and compared their resistance profiles by comprehensive mutational analysis. SC34 and SC34EK selected several mutations within the gp41- and gp120-coding sequences over a period greater than 1 year. Phenotypic and replication kinetics analyses revealed that in the case of both inhibitors, changes in gp41 sequences served as primary mutations that decreased resistance to the inhibitors, whereas the changes in gp120 were secondary mutations compensatory in nature. However, the mutated regions of gp41 of the selected SC34- and SC34EK-resistant viruses were considerably different; mutations selected by SC34 were

Resistance Profile of SC34 and SC34EK

mostly located within N-HR and C-HR, whereas more than half of those by SC34EK were located in another region of gp41. The molecular mechanisms and interactions that determine the effects of gp41 mutations outside the HRs on HIV-1 replication kinetics and the fusion process are not well understood. Therefore, further biological and structural studies focused on such interactions may reveal novel insights into the mechanism of fusion and the inhibition by drugs that target HIV-1 fusion.

SC34EK was designed to possess unidirectionally aligned EK pairs by modifying SC34 to have the two reverse-oriented EK pairs (Fig. 1). Thus, the difference in peptide sequences induced a different resistance pattern and reduced cross-resistance. Although accumulation of multiple mutations in gp41 eventually conferred a high level of resistance to SC34 and SC34EK, susceptibility to both inhibitors was not significantly affected by single amino acid substitutions. Moreover, it has been reported that substitutions in gp120 modulate the susceptibility to T-20 (33–36), and we also observed that both SC34- and SC34EK-selected mutations in the gp120 conferred resistance to T-20 but not to SC34 and SC34EK. These results indicate that SC34 and SC34EK have a high genetic barrier of resistance.

One mutation appeared to contribute significantly to the reduced cross-resistance of SC34- and SC34EK-resistant variants. Specifically, SC34EK selected the N43K mutation, whereas T-20 selected for N43D (31, 37), which is frequently observed together with E137K, a compensatory mutation in C-HR that maintains interaction with the N43D-substituted N-HR, possibly through the Asp⁴³-Lys¹³⁷ ion pair (38, 39). SC34EK has been designed to harbor Lys¹³⁷ in its peptide sequence. Hence, it is possible that resistance to SC34EK emerges through unfavorable repulsive charge-charge interactions between the Lys⁴³ of the viral N-HR and the Lys¹³⁷ residue of SC34EK. Therefore, charge interactions between amino acids 43 and 137 are likely to be the mechanism for resistance to T-20 and in part to SC34EK, but not to SC34, which has a glutamic acid (Glu⁴³) at this position. This might be one of the reasons why SC34 and SC34EK did not show significant cross-resistance.

We recently demonstrated that T-20_{S138A} and C34_{N126K} are able to suppress T-20- and C34-resistant variants, respectively (18). We again applied the same strategy and introduced resistance mutations in SC34 and SC34EK and examined the effect of these changes on their potency against SC34- or SC34EK-resistant variants. In this case, only mutations in the C-HR conferred enhanced susceptibility by augmenting binding affinity to the target N-HR. In the case of the C34_{SC34EK}^{res} peptide, although we expected minimal impact of H132Y on drug susceptibility and/or on C-HR conformation, S138A appeared to stabilize the 6-helix complex by improving hydrophobic contacts with the pocket formed by Leu⁴⁴ and Leu⁴⁵, as reported previously (18). In the case of the C34_{SC34}^{res} peptide, in addition to the aforementioned S138A effect, it was expected that N126K would enable the formation of possible intra-helical salt bridges with Glu¹²³ that would stabilize the α -helicity of C-HR. These mutations im-

proved the anti-HIV-1 activity toward wild-type, and surprisingly, to SC34EK-resistant variants as well.

A number of potent peptide fusion inhibitors that suppress T-20-resistant variants have been previously reported (13, 14, 40, 41). Resistance profiles of these next generation fusion inhibitors with physicochemical modifications are expected to be different from those of the native sequence peptide fusion inhibitors, although only those of T-1249 and T-2635 were examined (42, 43). T-1249, one of the first next generation fusion inhibitors, showed potent anti-HIV-1 activity in HIV-1-infected patients that failed to respond to T-20 treatment (12). However, mutations at positions 36–45 (such as V38A/E, Q40H/K, and N43D/K), which are also observed in T-20-resistant variants *in vitro* and *in vivo*, were also detected in clinical trials of T-1249 (12, 44, 45). Nearly all the individual selected mutations had little impact on the susceptibility to T-1249. However, V38D/E conferred high-level resistance to T-1249 (30-fold) and T-20 (more than 200-fold), but not to another fusion inhibitor, T-2635 (42, 43), suggesting that there is potential cross-resistance between T-20 and T-1249. In contrast, T-2635 was hardly affected by such single mutations except for Q79E and K90E with a mild resistance of 4- and 7-fold, respectively (42), indicating that T-2635 had a preferential resistance pattern similar to SC34 and SC34EK, because these inhibitors were essentially effective against all variants with single mutations. Interestingly, although HIV-1_{SC34(P-122)} and HIV-1_{SC34EK(P-120)} showed mild (7.3-fold) and moderate resistance (21-fold) to C34, respectively, we observed significant differences in resistance against T-2410, another next generation fusion inhibitor, which differed from C34 by only two added amino acids at each of the N and C termini (13). Specifically, the susceptibilities of HIV-1_{SC34(P-122)gp41} and HIV-1_{SC34EK(P-120)gp41} to T-2410 were decreased by 38- and 3.7-fold, respectively. Although the mechanism underlying the ineffectiveness of T-2410 against SC34-resistant HIV-1 remains unclear, it appears that the size of the peptide inhibitor may be another parameter that should be considered in future attempts to design fusion peptide inhibitors with improved resistance profiles. Meanwhile, sifuvirtide and T-20EK did not show anti-HIV-1 activity against either SC34- or SC34EK-resistant variants, suggesting that they may partially share a common resistance profile. Sifuvirtide was designed based on the sequence of the C34 region of gp41 derived from HIV-1 subtype E. Similar to SC34 and SC34EK, sifuvirtide includes amino acid substitutions that could form intramolecular salt bridges. However, the majority of amino acids in sifuvirtide must be bound to the same region of N-HR, where C34, SC34, and SC34EK may interact (14). This may explain why sifuvirtide was inactive against SC34- and SC34EK-resistant variants. T-20 derivatives, including T-20EK, lack the N-terminal tryptophan-rich domain (N-TRD), also known as the pocket-binding domain, that interacts with the hydrophobic groove of the N-HR trimer (46), but they have the C-terminal TRD that interacts with the lipid bilayer at the cellular membrane (47). Our results showed that T-20-derived peptides seem less active compared with C34 derivatives with N-TRD. Recently, treatment with two or three fusion inhibitors was reported to have a potent and syn-

ergistic antiviral activity on T-20-resistant variants (48, 49). Together, these observations indicated that each inhibitor has a distinct inhibitory mechanism that may lead to the design of a combination therapy of fusion inhibitors *in vivo*.

In conclusion, the barrier for resistance to SC34 and SC34EK is considerably higher than that for the parent compound C34, or for T-20. Moreover, these inhibitors have a distinct resistance profile from C34, T-20, and other next generation fusion inhibitors. Hence, they are excellent alternatives for clinical use. Although mutations induced by SC34 and SC34EK are partially overlapped, most mutations were specific to each agent. Importantly, we demonstrated that interchange of only two pairs of EK positions could reduce cross-resistance. Because sites and direction of the EK modification seem to be easily replaceable, this is a useful strategy to suppress more efficiently emergence of resistant variants. Moreover, the present study demonstrates that the usefulness of this strategy that we have previously applied to design improved fusion inhibitors with HIV-1 sequences (18) has been extended to improve fusion inhibitors with "artificial" (non-HIV) sequences.

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