Table 3
Antiviral activity of HIV-1 gp41-derived peptides against HIV-2[a]

HIV-2 strain	EC_{50} (nM)				
	ddC	T-20	C34	SC34	SC34EK
WT[b]	404 ± 196	35 ± 17	3.2 ± 0.9	1.4 ± 0.7	0.7 ± 0.3
HIV-2_{EHO}[c]	925 ± 188	14 ± 3.0 (×0.4)	**639 ± 87 (×200)**	**68 ± 10 (×49)**	**17 ± 1.2** (×24)
HIV-2_{ROD}[d]	**1808 ± 927**	**176 ± 68 (×5)**	**>1000 (>×313)**	**251 ± 29 (×179)**	**115 ± 33** (×164)

[a] Anti-HIV-2 activity was determined using the MAGI assay. All data represent mean ± standard deviation obtained from the results of three independent experiments. Bold indicates over 5-fold increase in EC_{50} value compared to HIV-1_{WT}.
[b] HIV-1_{NL4-3} served as a wild-type virus.
[c] HIV-2_{EHO} was dual-tropic HIV-2.
[d] HIV-2_{ROD} was T-tropic HIV-2.

various cryo-conditions, the suitable condition was found to be the addition of 35% xylitol to the peptide solution and a slight increase in the amount of the precipitant (*ca* 14.5%). The obtained crystals were easily broken by direct transfer from the crystallization condition to the cryo-condition, but the transfer of the fragile crystals could be accomplished by gradual change in conditions using stepwise increase in the amount (0–35% in five steps) of the cryoprotectant.

Data were collected at a beamline BL38B1 of SPring-8. Collected data were processed using DENZO and SCALEPACK from the HKL2000 package (Otwinowski and Minor, 1997). A molecular replacement solution was found using AMoRe (Navaza, 2001), with a molecular model of the HIV-1 gp41 core structure (PDB code: 1AIK). Model refinements and reconstruction were performed using REFMAC5 (Murshudov et al., 1999) and XtalView (McRee, 1999). The final model was refined at a resolution of 2.1 Å, to a crystallographic *R* value of 0.213 and a free *R* value of 0.238. Detailed data collection and refinement statistics are summarized in Table 1. Atomic coordinates and structural factors have been deposited at the Protein Data Bank (PDB code:2Z2T).

3. Results

3.1. Anti-HIV-1 activity of SC34 and SC34EK

We examined the anti-HIV-1 activity of SC34 and SC34EK against not only HIV-1_{WT} but also T-20- and/or C34-resistant clones observed in vitro. SC34 and especially SC34EK that has aligned EK modification more effectively suppress HIV-1 infection compared to C34 and T-20 (Table 1). D36S/V38M substitutions in the gp41 region (HIV-$1_{D36S/V38M}$), and a five amino acid (FNSTW) deletion in the V4 region of gp120 (ΔV4) with L33S/N43K in the gp41 region (HIV-$1_{\Delta V4/L33S/N43K}$) were isolated in vitro (Fikkert et al., 2002; Rimsky et al., 1998). L33S was also selected during C34-resistant induction in vitro (Armand-Ugon et al., 2003). C34 and its derivatives effectively inhibit entry of these clones into the host cell. In particular, SC34EK maintained strong activity even against V38E containing clones, such as HIV-$1_{V38E/N42S}$ (Armand-Ugon et al., 2003), which showed cross-resistance to T-20, C34 and SC34. Reduction of activities by SC34 and SC34EK was moderate in HIV-$1_{\Delta FNSTW/L33S/N43K}$ that showed high level resistance to T-20 and C34. Next, we examined the antiviral activities of C34 derivatives against clones containing major primary mutations V38A and N43D, which are mutations frequently observed in T-20 resistant variants in vivo (Cabrera et al., 2006; Derdeyn et al., 2001; Menzo et al., 2004; Poveda et al., 2004; Poveda et al., 2002; Xu et al., 2005) (Table 1). SC34 reduced its antiviral activities against HIV-1_{N43D}, while SC34EK maintained its potent activity, indicating that when EK is bound with the complementary electrostatic interactions appropriately aligned SC34EK can effectively suppress the infection by various clones resistant to T-20 and C34 both in vitro and in vivo.

We further evaluated activities of SC34 and SC34EK against V3-substituted clones (Table 1). HIV-$1_{V3\text{-}ADA}$ uses mainly the CCR5 co-receptor for its entry into the host cells and has been reported to moderate T-20 resistance (≈10 fold), compared to the CXCR4 using strain of HIV-1, which shows higher susceptibility to fusion inhibitors (Reeves et al., 2002). As reported, the susceptibility of HIV-$1_{V3\text{-}ADA}$ to T-20 decreased, however, C34 and its derivatives maintained their activity against the same variant. Interestingly, in our experiments, HIV-$1_{V3\text{-}SF162}$, HIV-$1_{V3\text{-}CH1}$ and HIV-$1_{V3\text{-}CH2}$ also showed comparable susceptibility to T-20. These results indicate that sequence variations in the V3 region do not always correlate with the observed T-20 susceptibility and are not involved in the resistance to C34 and its derivatives.

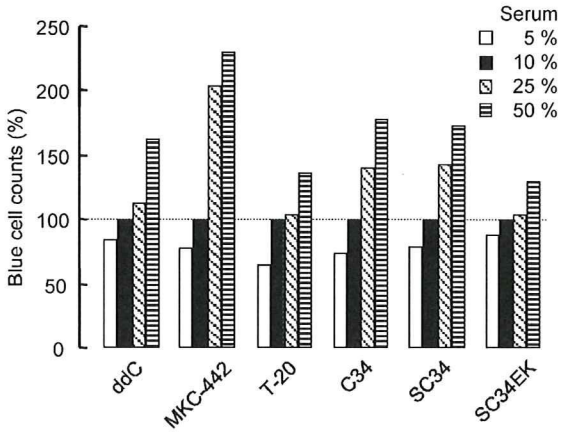

Fig. 3. Effect of FCS concentrations on anti-HIV-1 activity. Changes in the blue cell counts at various concentrations of FCS are shown. Blue cell counts at EC_{80} value in 10% FCS concentration (black bar) were used and set as 100%. White, black, hatched, and striped bars correspond to 5, 10, 25, and 50% FCS, respectively. Inhibitors for reverse transcriptase, ddC and MKC-442, and for fusion, T-20 were used as controls.

3.2. Amino acid sequence

Amino acid sequences of clinical isolates are shown in Fig. 2b. One isolate, HIV-1_{KT}, was obtained from a drug-naïve patient and the other three isolates (HIV-1_{411}, HIV-1_{412}, HIV-1_{A03}) were obtained from heavily drug-experienced patients. None of the patients had received T-20 therapy. Amino acid sequences of the N-HR were highly conserved within all HIV-1 clinical isolates with some small variations. In contrast, the N36 region of the two HIV-2 strains, EHO and ROD, was identical in both HIV-2 isolates. We found some variations in the amino acid sequences of the HIV-2 strains we isolated, as compared with the sequences deposited in the GenBank (accession number; M15390 and X05291 for HIV-2_{ROD}, and U272000 for HIV-2_{EHO}). Namely, we identified two different amino acids in the isolated HIV-2_{ROD}, V26L and

I157I/M (mixture of I and M), and one variation in the amino acid sequence of HIV-2_{EHO}, V45L. Except for I157M, other substitutions are observed in the majority of the HIV-2 strains, as reported in the HIV sequence database (Los Alamos National Laboratory: Los Alamos, NM, USA, http://www.hiv.lanl.gov). These substitutions might be introduced through different culture conditions, (e.g., host cells used for the propagation). We considered these substitutions as a polymorphism.

Sequence homology of the N36 region of the isolated HIV-1 strains was 31/36 (86%), including mutation D36G that is observed in the vast majority of HIV-1 strains (Kuiken et al., 2001). In contrast, those of the C34 region were relatively heterogeneous, 24/34 (71%) for HIV-1 and 12/34 (35%) for HIV-2. Sequence identity of the T-20 region (residues 117–152) in the HIV-1 strains was also variable 27/36 (75%), while in the HIV-2 strains the sequence identity was 15/36 (42%). These results indicate that even highly conserved two helical extracellular domain of the gp41 can allow polymorphisms.

3.3. Efficacy of the peptides against clinical isolates

To evaluate preclinical efficacy, we examined the antiviral activity of C34, SC34 and SC34EK against clinical isolates (Table 2). Replication of HIV-1_{NL4-3} and HIV-1_{KT}, a drug-naïve strain, was suppressed by all compounds tested. C34 showed decreased activity against HIV-$1_{IVR-A03}$, which was isolated from a heavily drug-exposed patient. SC34 also showed reduced susceptibility against three drug-experienced strains. However, it is difficult to conclude whether SC34 showed enhanced susceptibility against HIV-1_{KT} or reduced susceptibility against drug resistant strains. In contrast, T-20 and SC34EK suppressed the replication of all isolates tested to similar extents in EC_{50} values compared to HIV-1_{NL4-3} (Table 2), indicating that SC34EK with appropriately aligned EK residues effectively suppresses the replication of the clinical 3 isolates.

3.4. Anti-HIV-2 activity

To confirm the target specificity, we examined antiviral activities of SC34 and SC34EK against two HIV-2 strains, EHO and ROD. Compared to HIV-1_{NL4-3}, EHO and ROD contain 19 and 22 amino acid substitutions in the C34 region, respectively, and 15 amino acid substitutions in the N36 region, the anticipated site of binding of SC34 and SC34EK peptides (Fig. 2b). Like the parent peptide C34, both SC34 and SC34EK lost their potent activities (Table 3). Compared to HIV-1_{NL4-3}, 6 out of 19 residues in the C34 region of HIV-2_{EHO} and 7 out of 22 residues in the C34 region of HIV-2_{ROD} are located at positions *a*, *d*, and *e* that directly interact with the N36 binding surface. These substitutions in the N36 and C34 region in HIV-2 may be responsible for reduced anti-HIV-2 activities of the peptides derived from HIV-1. At present, we cannot conclude which amino acid substitutions are directly involved in the reduced susceptibility of the HIV-2 strain to the treatment with the peptide fusion inhibitor, and/or whether other regions besides the N36 and C34 regions might influence peptide susceptibility. However, our results indicate that SC34 and SC34EK maintain similar target specificity to the parent peptide, C34.

3.5. Effect of fetal calf serum (FCS) on anti-HIV-1 activity

To estimate the stability of the peptides in vivo, binding level of SC34EK, to serum components, (e.g., albumin) was examined. In this experiment, the antiviral activity in the presence of relatively high concentrations of fetal calf serum (FCS) was determined (Baba et al., 1993) (Fig. 3). EC_{80} values of the fusion inhibitors against HIV-1 replication in vitro were used. In the presence of 50% FCS, the activity of MKC-442 (I-EBU), a lipophilic non-nucleoside RT inhibitor, was reduced 2.3-fold compared with 10% FCS as described previously (Baba et al., 1993). However, the activities of SC34, SC34EK and T-20 were little influenced by serum components. Among the three, SC34EK was the least affected by the concentration of FCS.

We further examined the stability of peptide inhibitors in freshly prepared human sera ($n = 3$). After 1 h incubation of peptides in human sera (final concentration of 200 μM) at 37 °C, the anti-HIV-1 activity was examined using the MAGI assay. Comparable activities of all peptides tested were observed either with or without the incubation (data not shown). These results indicate that hydrophilic SC34EK likely retains its strong anti-HIV-1 activity in vivo, similarly to T-20, because of its low non-specific binding and protease cleavage in serum.

3.6. Peptide binding affinity

To clarify the mechanism of potent anti-HIV-1 activity observed with SC34EK, the binding affinity of SC34EK was evaluated by collecting the CD spectra using synthetic peptides. The CD spectra of equimolar mixtures of the N-HR and C-HR peptides showed spectrum minima at 208 and 222 nm, which indicate the presence of stable α-helical conformations. All combinations of peptides showed similar spectra at 25 °C, indicating that these peptides contained the same α-helicity (Fig. 4a), although the spectrum of C34 with N36 and N43D mutation ($N36_{N43D}$) indicated only weak α-helicity. These results indicate that N43D might reduce the stability of the conformation of the 6-helix bundle, thus decreasing the replication of HIV-1, whereas V38A does not. SC34EK formed stable 6-helix conformations with $N36_{V38A}$ and $N36_{N43D}$. Under these experimental conditions, wavelength-dependent spectra were similar with the exception of the spectrum of the $N36_{N43D}$/C34 complex. Thus, we analyzed thermal stabilities, defined as the midpoint of the thermal unfolding transition (T_m) values, of the potential 6-helix bundles of N-HR and C-HR peptides. T_m of N36/C34 was found to be 52.0 °C, while that of $N36_{V38A}$/C34 and $N36_{N43D}$/C34 decreased to 44.5 and 34.0 °C, respectively (Fig. 3b). In contrast, thermal stabilities of $N36_{V38A}$/SC34EK, $N36_{N43D}$/SC34EK and N36/SC34EK were much higher, 60.5, 56.0 and 69.5 °C, respectively. Thus, binding affinity of SC34EK to N-HR was stronger compared to that of C34. Alternatively, at the physiological temperature of 37 °C, only 60 and 40% of the α-helix content was observed in $N36_{V38A}$/C34 and $N36_{N43D}$/C34 mixtures, respectively, indicating that roughly half of C34 failed to form stably 6-helix bundle with the target N-HR harboring resistant mutations. Therefore, C34 reduces its anti-fusion activity exerted by dominant negative effect. In contrast, only 20% of the unfolded α-helix content was observed in SC34EK with mutated N36, which indicated that at 37 °C, binding of SC34EK to mutated N36 was comparable to that of C34 with wild-type N36 (Fig. 4b). Moreover, physiochemical properties of N-HR and SC34EK complexes, defined by T_m value, correlated well with their ability to inhibit HIV-1 fusion (Fig. 4c). These results suggest that the stability of the 6-helix complex, as judged by the binding stability (affinity), is directly correlated with the anti-HIV-1 activity.

3.7. Crystal structure of the N36/SC34EK complex

The crystal structure of the complex between SC34EK and the N-HR representative peptide N36 was resolved to a resolution of 2.1 Å (Table 4). In the asymmetric unit, a 6-helix bundle consisting of a central helix bundle of three N36 peptides surrounded by three SC34EK peptides was found. This arrangement is similar in the core structure of gp41 (Chan et al., 1997). Structural superimposition of the original gp41 core and the N36/SC34EK complex showed a good match, with an RMSD value of 0.59 for main-chain atoms

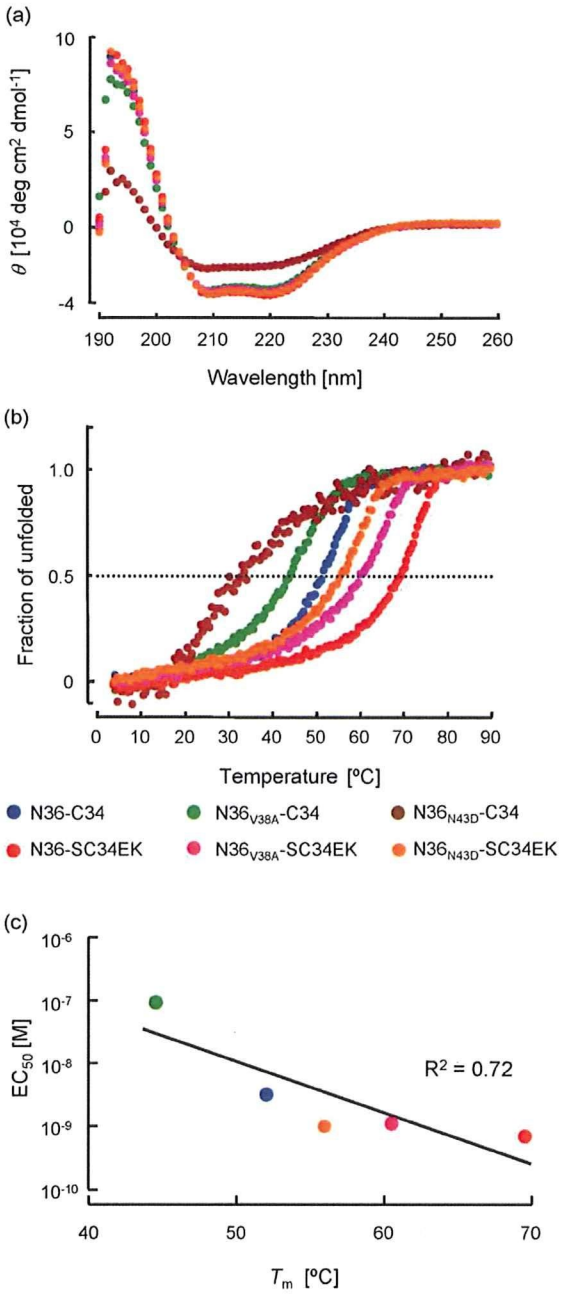

Fig. 4. CD analysis of peptide complex between resistant variants of N36 and C34 or SC34EK. (a) Wavelength-dependent CD spectra of the complexes in solution. The spectrum minima at 208 and 222 nm indicated the presence of stable α-helical conformations. (b) Thermal midpoint analysis was measured at 222 nm CD signal for the N and C peptide complexes. Final concentration of each peptide was 10 mM. The arrow indicates the physiological temperature of 37 °C. (c) The correlation between T_m (b) and EC_{50} values (Table 1). Colors of plots correspond to those in panels (a) and (b). Combination of $N36_{N43D}$ and C34 (EC_{50} > 100 nM) is excluded.

(Fig. 5a and b). Hydrophobic contacts between SC34EK and N36 with tryptophan rich domain (WRD) and leucine zipper were preserved for the original gp41 core. All introduced charged residues of the EK motif were directed toward the solvent (Fig. 5c). As a direct consequence of introducing the EK motifs, the ratio of surface area occupied by charged residues to the total surface area was increased from 35% in the original molecule to 60% in the N36/SC34EK complex. Importantly, it appeared that tight bonding, such as ion pairing or hydrogen bonding, was not present in the

Table 4
Crystallization, data collection and refinement statistics

Data collection	BL38B1 Spring-8
Temperature (K)	100
Space group	$P3_121$
Cell dimensions *a*, *b*, *c* (Å)	105.01, 105.01, 78.31
Resolution limits (Å)	90.00–2.10
Number of unique reflections	29,461
Average redundancy	7.53
Completeness (%)	99.7
R_{merge} [a]	0.122
Refinement statistics	
Refinements resolution range (Å)	20.00–2.20
R/R_{free} [b] (%)	0.213/0.238
The highest resolution shell (Å)	2.15–2.10
R/R_{free} [b] (%)	0.231/0.255
RMSD from ideal	
Bonds (Å)	0.010
Angles (°)	1.015
⟨*B*⟩ for atomic model [c] (Å²)	29.93
Ramachandran plot	
Most favored regions (%)	100

[a] $R_{merge} = \sum|I_h - \langle I_h\rangle| / \sum I_h$, where $\langle I_h\rangle$ is the average intensity of reflection *h* and symmetry-related reflections.

[b] *R* and $R_{free} = \sum||F_o| - |F_c|| / \sum|F_o|$ calculated for reflections of the working set and test (5%) set, respectively.

[c] ⟨*B*⟩ is the average temperature factor for all protein atoms.

side-chains of the residues of the EK motif. Electrostatic interaction may involve in constrained structure which provides the enhanced α-helicity observed (Fig. 4). This structural analysis demonstrated that the interaction between N36 and SC34EK retained the ability to form the 6-helix bundle structure despite the substitution of more than one third of the residues (13/34) in the sequence of SC34EK.

4. Discussion

In this study, we characterized a novel α-helical peptide, SC34EK that effectively inhibits replication of HIV-1 strains resistant to T-20 and C34. The activity was specific to HIV-1 and little influenced by serum components. We demonstrate that the potent anti-HIV-1 activity of SC34EK is derived from its high affinity to the N-HR region by the CD analysis. Further, we reveal that SC34EK binds to its target, N-HR in identical manner that C34 does by the structure analysis.

The structural analysis of the N36/SC34EK complex clearly demonstrated that the interaction between SC34EK and N36 peptides was maintained by hydrophobic contacts and that the EK motif was directed toward the solvent. The introduction of the EK residues increased the proportion of accessible surface area occupied by charged residues. Although tight bonding was not observed, a continuous electrostatic potential between the EK residues may serve to stabilize the helix bundle. Such helix stabilization, which might occur on the surface of the HIV-1 virion between SC34EK and the N36 region of gp41, could result in the high anti-HIV-1 activity. In this regard, SC34EK, containing an aligned EK motif, showed more potent anti-HIV-1 activity compared to SC34, which has one misaligned EK motif (Fig. 2a). Increasing the hydrophilic surface area may prevent aggregation of SC34EK as compared to parental peptide C34. Therefore, SC34EK might distribute into the various organs in the body without being trapped and destroyed in the reticular systems or having its activity reduced by non-specific binding to proteins (e.g., albumin) (Fig. 3).

We further demonstrate that SC34EK specifically binds to the target, N-HR of HIV-1, since it only exerted weak activity to two

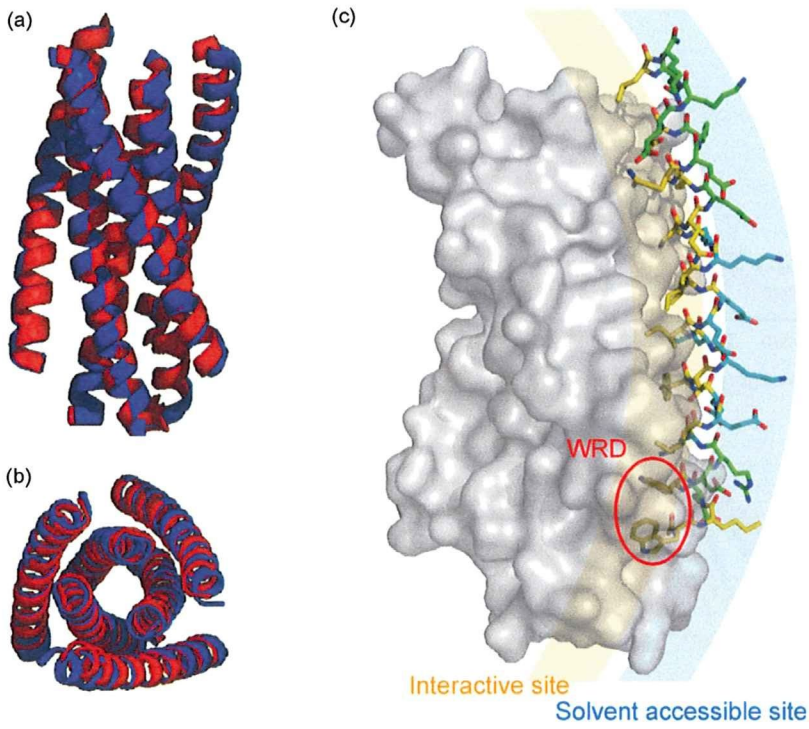

Fig. 5. Structure of the 6-helix bundle formed by N36 and SC34EK. (a and b) The gp41 core structure and N36/SC34EK complex are shown in red and blue, respectively. (c) Stick model representation of SC34EK. The stick model of SC34EK is shown, and three N36s in the core and two other surrounded SC34EK are represented in gray. SC34EK showed amphiphilic properties. The location of the N-terminal tryptophan rich domain (WRD) in SC34EK is indicated by a red circle. Original and introduced charged amino acids are indicated in green and blue, respectively.

HIV-2 strains that contain 15 amino acid substitutions in the N-HR compared to HIV-1 NL4-3 strain (Table 3 and Fig. 2b). These results suggest that to develop resistance to SC34EK, at least, certain mutations in not only the N-HR but also the C-HR are required to be introduced. This might delay emergence of resistant HIV-1 variants to SC34EK in vivo.

So far, some approaches for stabilizing α-helix structures through the introduction of artificial amino acids were reported for HIV-1 fusion inhibitors T-20 (Judice et al., 1997) and C34 (Sia et al., 2002), including an example of an amino acid containing terminal olefin-derived side chains, designed as a substrate for the ring-closing olefin metathesis (Blackwell et al., 2001) and an example of a hydrocarbon-stapled peptides (Phelan et al., 1997) Walensky et al. (2004) applied a hydrocarbon-stapled modification to generate peptides that bind to the BH3 helical domain of Bcl-2, an anti-apoptotic protein, and demonstrated that a synthesized peptide mimic that binds to the BH3 domain activates apoptosis in leukemic cells. However, all peptides exerted only moderate activity in vivo, although they showed efficient binding to the target proteins in vitro (Blackwell et al., 2001; Judice et al., 1997; Sia et al., 2002; Walensky et al., 2004). It is likely that during the formation of the 6-helix bundle and the fusion process, gp41 changes its conformation drastically, suggesting that a flexible conformation of the peptide may be required to preserve actual inhibition. Compared with tethered, constrained peptides, EK modification that facilitates electrostatic stabilization displays such flexibility while exhibiting enhanced α-helicity. Most recently, T290676, a 38 amino acid peptide, has been reported to suppress various fusion inhibitor-resistant strains of HIV-1 (Dwyer et al., 2007). Like SC34EK, T290676 is substituted with the charged and hydrophilic amino acids, glutamic acid (E) and arginine (R), at the solvent accessible site and shows potent anti-HIV-1 activity.

In conclusion, we have demonstrated that SC34EK selectively inhibits various HIV-1 strains, including T-20 resistant clones, through increased stability of the α-helix. The sequence of the solvent accessible site of α-helical peptides is replaceable and modifications of this sequence can regulate α-helicity with target specificity. Therefore, our approach of introducing the EK motif in the α-helical structure of the peptide inhibitor will help to generate future peptide inhibitors with high anti-HIV efficacy and potentially fewer adverse effects.

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Design of Peptide-based Inhibitors for Human Immunodeficiency Virus Type 1 Strains Resistant to T-20*[S]

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Enfuvirtide (T-20) is a fusion inhibitor that suppresses replication of human immunodeficiency virus (HIV) variants with multi-drug resistance to reverse transcriptase and protease inhibitors. It is a peptide derived from the C-terminal heptad repeat (C-HR) of HIV-1 gp41, and it prevents interactions between the C-HR and the N-terminal HR (N-HR) of gp41, thus interfering with conformational changes that are required for viral fusion. However, prolonged therapies with T-20 result in the emergence of T-20-resistant strains that contain primary mutations such as N43D in the N-HR of gp41 (where T-20 and C-HR bind) that help the virus escape at a fitness cost. Such variants often go on to acquire a secondary mutation, S138A, in the C-HR of gp41 region that corresponds to the sequence of T-20. We demonstrate here that the role of S138A is to compensate for the impaired fusion kinetics of HIV-1s carrying primary mutations that abrogate binding of T-20. To preempt this escape strategy, we designed a modified T-20 variant containing the S138A substitution and showed that it is a potent inhibitor of both T-20-sensitive and T-20-resistant viruses. Circular dichroism analysis revealed that the S138A provided increased stability of the 6-helix bundle. We validated our approach on another fusion inhibitor, C34. In this case, we designed a variant of C34 with the secondary escape mutation N126K and showed that it can effectively inhibit replication of C34-resistant HIV-1. These results prove that it is possible to design improved peptide-based fusion inhibitors that are efficient against a major mechanism of drug resistance.

HIV-1[2] entry into the target cells is mediated by two envelope glycoproteins, gp120 and gp41, that form a trimeric gp120·gp41 complex. After binding of gp120 to the CD4 receptor and CCR5 (or CXCR4) coreceptor on the surface of the target cell, 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 an intermediate that is anchored to both cellular and viral membranes. After this step, the 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) (1, 2). This refolding brings the viral and cellular membranes together to catalyze fusion.

The transition of the extended intermediate to the 6-helix bundle can be inhibited by the addition of exogenous peptides derived from gp41 C-HR (Fig. 1*A*) that prevent the formation of the 6-helix bundle and inhibit the HIV-1 fusion with the target cells (3–6). T-20, a 36-amino acid peptide derived from C-HR, effectively suppresses *in vivo* replication of HIV-1 resistant to inhibitors of reverse transcriptase and protease (7, 8). However, HIV-1 variants resistant to T-20 have recently emerged carrying primary mutations in the Leu-33–Leu-45 region of the N-HR domain (9–15). Among them, V38A and N43D seem to be major primary mutations for T-20 resistance. Meanwhile, a secondary mutation at the C-HR region (S138A) has been reported to enhance T-20 resistance with an as yet undefined mechanism (9, 14, 15) (Fig. 1*B*).

The mechanism of resistance to C34, another C-HR peptide-based inhibitor of HIV fusion, has been the subject of multiple studies (13, 16). Because of a 22-amino acid overlap between the T-20 and C34 peptides (Fig. 1*B*), HIV-1 has developed primary mutations for C34 resistance *in vitro* at the identical Leu-33–Leu-45 region of the peptides. During *in vitro* selection of C34 resistance, we identified a mutation in the C-HR domain, N126K, that is also observed in some T-20-resistant clinical variants (10, 15, 17). We showed that N126K conferred resistance to C34 by compensating for the impaired intra-gp41 inter-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2 and Tables 1 and 2.

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[2] The abbreviations used are: HIV, human immunodeficiency virus; T-20, enfuvirtide; HR, heptad repeat; MAGI, multinuclear activation of galactosidase indicator; EC_{50}, 50% effective concentration; T_m, melting temperature; CD, circular dichroism; shRNA, short hairpin RNA; WT, wild-type.

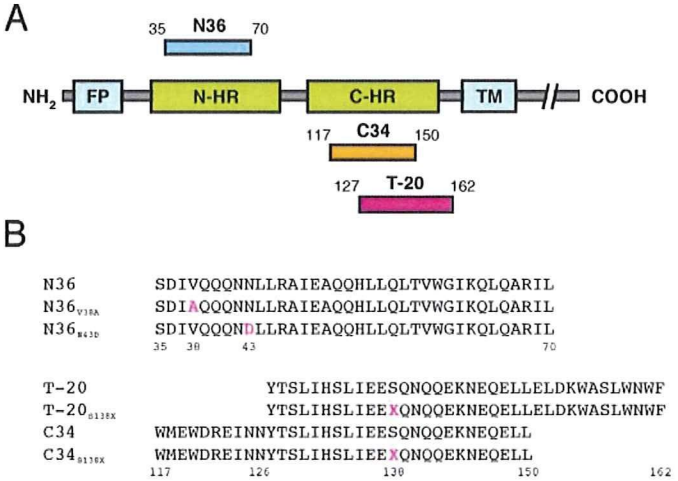

FIGURE 1. **Schematic view of gp41 and peptide sequence.** *A*, structure of HIV-1 gp41 and locations of N-HR or C-HR peptides (*FP*, fusion peptide; *TM*, transmembrane domain). *B*, amino acid sequences of peptides used in this study. Only the amino acid located at Ser-138 was substituted with all physiological amino acids (*X*), as Asn-126 lies outside of the amino acid sequence of T-20.

action by a primary mutation, I37K (13). N126K was initially identified in background of V38A, another primary mutation, for T-20 resistance *in vivo* (17). Baldwin *et al.* (17, 18) demonstrated a striking T-20-dependent replication phenotype in the V38A/N126K variant and proposed that T-20 acts as a safety pin to prevent premature formation of helical bundle, as N126K enhanced binding capacity of the introduced C-HR to N36 with V38A. Taken together, these studies suggest that mutations in the C-HR serve as secondary mutations.

In this study we show that the main role of secondary mutations that follow the appearance of primary mutations during treatment with peptide-based fusion inhibitors is to compensate for the impairment in replication kinetics that is caused by the primary mutations (supplemental Fig. 1). Based on this finding we hypothesized that analogs of T-20 carrying substitutions corresponding to secondary T-20 resistance mutations should be active against both wild-type and T-20-resistant viruses containing primary mutations. Indeed, our results confirmed our hypothesis and showed that T-20 with the S138A substitution ($T\text{-}20_{S138A}$) has a strong anti-HIV-1 activity even against T-20-resistant clones. Moreover, we demonstrate that this restoration is concomitant to improved binding of $C\text{-}HR_{S138A}$ to $N\text{-}HR_{N43D}$, suggesting that our approach utilizing the resistance-associated mutations to design peptides may provide useful broad insights into effective peptide-based therapies.

EXPERIMENTAL PROCEDURES

Cells and Viruses—MT-2 cells were grown in RPMI 1640 medium. 293T cells were grown in Dulbecco's modified Eagle's medium-based culture medium. HeLa-CD4-LTR-β-gal cells were kindly provided by M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (Bethesda, MD) and were used for the drug susceptibility assay as described previously (13, 19, 20). An HIV-1 infectious clone, pNL4–3 (21), was used for generation of HIV-1 variants.

Antiviral Agents—The peptides used in this study were synthesized as described previously (6).

Determination of Drug Susceptibility of HIV-1—The peptide sensitivity of infectious clones was determined by the multinuclear activation of galactosidase indicator (MAGI) assay as described previously (13). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in 96-well flat 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, all the blue cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (50% effective concentration, (EC_{50}).

Generation of Recombinant HIV-1 Clones—Recombinant infectious HIV-1 clones, carrying various mutations, were generated as described previously (13). Each molecular clone was transfected into 293T cells with *Trans*IT® (Madison, WI). After 48 h, the supernatants were harvested and stored at −80 °C until use.

Circular Dichroism Spectroscopy—Each peptide (10 μM) was mixed with 10 mM phosphate-buffered saline, pH 7.4, and the data were collected using a Jasco spectrometer (Model J-710; Jasco, Tokyo, Japan) equipped with a thermoelectric temperature controller. The thermal stability was assessed by monitoring the change in the circular dichroism signal at 222 nm. The midpoint of the thermal unfolding transition (melting temperature, (T_m) of each complex was determined as described previously (6).

Viral Replication Kinetics Assay—MT-2 cells (10^5 cells/3 ml) were infected with each virus preparation (1000 MAGI unit) for 16 h. The infected cells were then washed and cultured in a final volume of 3 ml. The culture supernatants were harvested after infection on days 2–7, and the levels of p24 antigen were determined (22).

For each competitive HIV-1 replication assay, two infectious clones of interest that had been previously titrated were mixed and added to MT-2 cells (10^5 cells/3 ml) as described previously (13, 22) with minor modifications. To ensure that the two infectious clones being compared were of approximately equal infectivity, a fixed amount (500 MAGI unit) of one infectious clone was mixed with three different amounts (250, 500, and 1000 MAGI unit) of the other infectious clone. On day 1, one-third of the infected MT-2 cells were harvested and washed twice with phosphate-buffered saline, and the cellular DNA was extracted. The purified DNA was subjected to nested PCR and then direct DNA sequencing. The HIV-1 co-culture, which best approximated a 50:50 mixture on day 1, was further propagated. Every 3–4 days, the co-culture supernatant (100 μl) was transmitted to new uninfected MT-2 cells (5×10^5 cells/3 ml). The cells harvested at the end of each passage were subjected to direct sequencing, and the viral population change was determined.

Structure Modeling of gp41 S138A Mutant Core—The gp41 core model was built using the coordinates of crystal structure of the N36/C34 complex (23) (PDB code 1AIK). The coordi-

TABLE 1

Antiviral activity of T-20-derived peptides against T-20-resistant gp41 recombinant viruses

Anti-HIV activity was determined with the MAGI assay. The data shown are the mean values and S.D. that were obtained from the results of at least three independent experiments. Shown in parentheses are the -fold increases in resistance (increase in EC_{50} value) calculated by comparison to a reference virus. Increases of >10-fold are indicated in bold.

	EC_{50}			
	HIV-1_{WT}[a]	HIV-1_{V38A}	HIV-1_{N43D}	HIV-$1_{N43D/S138A}$
	nM			
T-20	2.4 ± 0.6	23 ± 8.2 (9.6)	49 ± 10 (**20**)	84 ± 16 (**35**)
Small				
T-20_{S138G}	1.3 ± 0.5 (0.5)	65 ± 8.8 (**27**)	141 ± 26 (**59**)	185 ± 68 (**77**)
T-20_{S138A}	0.6 ± 0.1 (0.3)	3.6 ± 1.7 (1.5)	3.5 ± 0.9 (1.5)	3.2 ± 1.0 (1.3)
Hydrophobic				
T-20_{S138V}	0.4 ± 0.2 (0.2)	31 ± 14 (**13**)	22 ± 3.5 (9.2)	23 ± 5.7 (9.6)
T-20_{S138L}	0.7 ± 0.1 (0.3)	13 ± 6 (5.4)	2.9 ± 0.7 (1.2)	2.2 ± 0.4 (0.9)
T-20_{S138I}	0.5 ± 0.1 (0.2)	4.9 ± 2 (2)	2.9 ± 0.8 (1.2)	2.4 ± 0.6 (1)
T-20_{S138M}	0.7 ± 0.2 (0.3)	4.4 ± 0.1 (1.8)	1.7 ± 0.5 (0.7)	1.2 ± 0.4 (0.5)
T-20_{S138P}	446 ± 167 (**186**)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)
Nucleophilic				
T-20_{S138T}	0.9 ± 0.2 (0.4)	39 ± 8.5 (**16**)	161 ± 35 (**67**)	124 ± 43 (**52**)
Aromatic				
T-20_{S138F}	9.4 ± 2.6 (**4**)	203 ± 89 (**85**)	393 ± 119 (**164**)	478 ± 116 (**200**)
T-20_{S138Y}	25 ± 9 (**10**)	516 ± 223 (**215**)	>1000 (>**416**)	>1000 (>**416**)
T-20_{S138W}	29 ± 14 (**12**)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)
Amide				
T-20_{S138N}	19 ± 4 (8)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)
T-20_{S138Q}	34 ± 11 (**14**)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)
Acidic				
T-20_{S138D}	210 ± 94 (**88**)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)
T-20_{S138E}	283 ± 80 (**118**)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)
Basic				
T-20_{S138H}	210 ± 85 (**88**)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)
T-20_{S138K}	708 ± 145 (**295**)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)
T-20_{S138R}	362 ± 114 (**150**)	>1000 (>**416**)	>1000 (>**416**)	>1000 (>**416**)

[a] To improve the replication kinetics, D36G mutation, observed in the majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (reference virus).

nates of the water molecules were removed. Additionally, the hydrogen atoms were placed in optimal positions and refined by the energy minimization with the AMBER9 program (24) using the FF99 force field. Ser-138 in the gp41 core model was replaced with alanine (replacement of -OH with -H), and the positions of the hydrogen atoms were refined as described above. The S138A mutant core model (N36/$C34_{S138A}$ complex) was further optimized by the energy minimization using the FF99 force field with the restraints on each of the three residues of N and C termini and the backbone atoms. The restraint weight was 5.0 kcal/mol Å^2.

RESULTS

Effect of Amino Acid Substitutions at 138 on Antiviral Activities—We chemically synthesized peptide analogs of T-20 with all natural amino acid substitutions at the 138 position (T-20_{S138X}) and evaluated them for their ability to inhibit three major T-20-resistant clones using the MAGI assay (13) (Table 1). The results indicated that only T-20_{S138A} inhibited replication of T-20-resistant clones as efficiently as the wild-type clone. Substitution to glycine enhanced T-20 activity, but unlike T-20_{S138A}, T-20_{S138G} reduced its activity against T-20-resistant clones by ~2–3-fold as compared with the parental peptide, T-20. Substitutions to hydrophobic amino acids leucine, isoleucine, and methionine maintained their anti-HIV-1 activity; however, those to valine reduced anti-HIV-1 activity to T-20-resistant clones. The proline substitution drastically decreased the anti-HIV-1 activity of the peptide inhibitors.

Nucleophilic amino acid at position 138 of T-20 (T-20_{S138T}) showed similar profiles. Conversely, aromatic and amide substitutions reduced the anti-HIV-1 activity of T-20 against HIV-1_{WT}- and T-20-resistant clones. Other amino acid substitutions, especially acidic and basic amino acids, decreased the anti-HIV-1 inhibitory activity even against HIV-1_{WT}. These results suggest that smaller hydrophobic (Ala > Leu, Ile) or more flexible (Met > Thr) residues are preferred in this position. Furthermore, the α-helical structure is important for the interaction, as a mutation to proline which is expected to disrupt the helix (25) resulted in an inactive T-20 analog.

Circular Dichroism—To clarify the mechanism by which the substitutions at Ser-138 influence the antiviral activity of T-20 derivatives, we examined the binding affinities of these peptides to N-HR using circular dichroism (CD) analysis (Fig. 2). CD spectra reveal the presence of stable α-helical structure of the 6-helix bundle that is a requisite for biological activity and is thought to be mechanistically and thermodynamically correlated with HIV-1 fusion (26). Therefore, CD spectra typically at 222 nm indicate interaction of N-HR (N36) and C-HR (T-20 or C34). Because T-20 does not interact significantly *in vitro* with the N36 peptide, which is derived from amino acids 35–70 of N-HR, we used a derivative of C34, a peptide that overlaps with T-20 and also inhibits HIV fusion by the same mechanism. The C34 derivative contained the analogous T-20 substitutions described above (Fig. 1*B*). Consistent with antiviral activities, a mixture of N36 and $C34_{S138P}$ or $C34_{S138W}$ showed no apparent or reduced α-helicity, respectively. For binding with $N36_{V38A}$

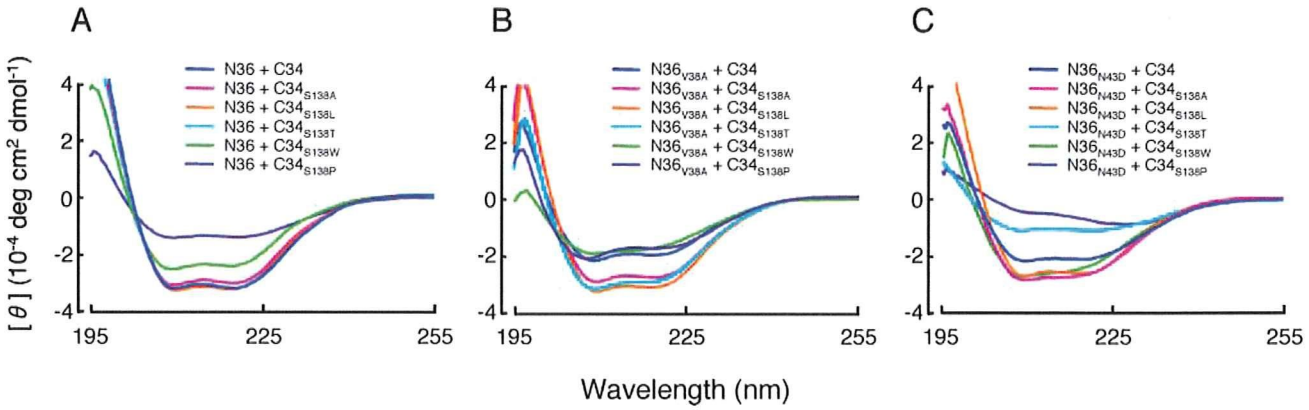

FIGURE 2. **CD spectra of $C34_{S138X}$ complexes with N36 (*A*), $N36_{V38A}$ (*B*), and $N36_{N43D}$ (*C*) are shown.** Equimolar amounts (10 μM) of the N- and C-HR peptides were incubated at 37 °C for 30 min in phosphate-buffered saline. The CD spectra of each mixture were then collected at 25 °C using a Jasco (Model J-710) spectropolarimeter.

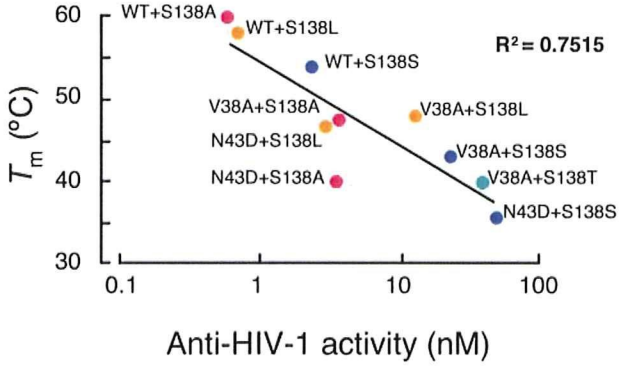

FIGURE 3. **Correlation of T_m values of complexes formed from N36 and C34 peptides (Fig. 2) and anti-HIV-1 activities of T-20_{S138X} (Table 1).**

TABLE 2

Antiviral activity of $C34_{N126K}$ peptides against C34-resistant gp41 recombinant viruses

Anti-HIV activity was determined by the MAGI assay. The data shown are the mean values and S.D. that were obtained from the results of at least three independent experiments. Shown in parentheses are the -fold increases in resistance (increase in EC_{50} value) calculated by comparison to a reference virus. The increase of >10-fold is indicated in bold.

	EC_{50}	
	HIV-1_{WT} [a]	HIV-$1_{\Delta V4/I37K/N126K/L204I}$ [b]
	nM	
C34	1.6 ± 0.35	114 ± 29 (**71**)
$C34_{N126K}$	0.95 ± 0.22 (0.6)	1.1 ± 0.5 (0.7)

[a] To improve the replication kinetics, the D36G mutation, observed in majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (reference virus).

[b] C34-resistant HIV-1 was constructed with the reference virus as described (13). ΔV4 indicates 5 amino acids deletion (FNSTW) in the V4 region of gp120.

or $N36_{N43D}$, sufficient α-helicity at 25 °C was observed only in $C34_{S138A}$, $C34_{S138L}$, and $C34_{S138T}$ or $C34_{S138A}$, $C34_{S138L}$, and $C34_{S138W}$, respectively (Fig. 2, *A–C*).

To determine the thermal stability of the helical complexes formed from the N36 and C34 peptides, we measured the melting temperature (T_m) of each complex (supplemental Table 1). The sigmoidal transition of the CD signal at 222 nm correlates with the thermal stability of the helical complexes formed from the N36 and C34 peptides, which in turn are indicative of the binding affinity of these peptides. The melting temperature (T_m) indicating the 50% disruption of 6-helix bundle was comparatively evaluated. Complexes of N36 and C34 containing the S138A or S138L substitutions (N36/$C34_{S138A}$ or N36/$C34_{S138L}$) showed high thermal stability, comparable with that of the wild-type N36/C34 complex. Similarly, the addition of the S138A or S138L also improved the thermal stability of the $N36_{N43D}$/C34 complex. These results reveal a striking correlation between the thermal stability and the anti-HIV-1 activity of the complexes (R^2 = 0.75, Fig. 3). The low T_m value of the complex formed from $N36_{N43D}$ and C34 suggests that virus containing the N43D mutation shows high resistance to T-20, likely due to less favorable thermodynamics that are expected to drive the formation of the 6-helix bundles containing T-20 inhibitor.

Antiviral Activity of Substituted C34 at Ser-138—To confirm that binding of C34 to N-HR is indeed representative of T-20 binding to N-HR, we examined the anti-HIV-1 activities of C34-derived peptides that have S138A substitutions. The $C34_{S138A}$ and $C34_{S138L}$ peptides showed potent anti-HIV-1 activities, similar to T-20_{S138A} and T-20_{S138L} (supplemental Table 2). Based on these findings, we conclude that the stability of complexes comprised of modified C34s and N36s containing T-20 resistance mutations offers a good measure of the binding affinity of T-20_{S138X} to N-HR.

Antiviral Activity of C34 with N126K—We have recently identified another mutation at the N-HR of gp41 (N126K) during exposure of HIV-1 to C34 *in vitro* (13). The N126K has been occasionally observed after prolonged T-20-containing therapy (10, 15). Here we have confirmed that the $C34_{N126K}$ peptide can also suppress a C34-resistant clone containing several mutations: I37K/N126K/L204I (Table 2). Therefore, peptides designed to have compensatory mutations seem to have potent antiviral activity. However, because residue 126 is located outside the amino acid sequence of T-20 (Fig. 1*B*), we could not examine the effect of N126K substitution on T-20 activity.

Replication Kinetics of Ser-138-substituted HIV-1—To evaluate the effect of Ser-138 substitutions on viral replication, we constructed molecular clones introducing several Ser-138 and determined their replication kinetics by measuring p24 gag antigen production in the culture supernatant. Single nucleotide changes to the TCA codon for Ser-138 may generate 4 amino acid substitutions, Ala, Thr, Leu, Pro, and Trp. As expected, the compensative substitution, S138A, in the T-20

resistance mutation N43D background enhanced replication kinetics of the N43D-containing clone as shown in supplemental Fig. 1. However, in the WT background the S138A appeared to decrease production of p24 as compared with HIV-1_{WT} (Fig. 4). Other substitutions also reduced their replication kinetics. Interestingly, the S138W substitution did not show measurable p24 production. Syncytia induction and single cycle replication kinetics of the Ser-138-substituted HIV-1 were also examined (supplemental Fig. 2). Sizes of syncytia of each virus formed in the MAGI cells (supplemental Fig. 2, *panels A–E*) were associated with p24-normalized single-cycle infectivities (supplemental Fig. 2, *panel F*) and multicycle replication kinetics (Fig. 4). These results suggest that substitutions at Ser-138 are not likely to appear in the absence of T-20 therapy or the emergence of N43D mutation.

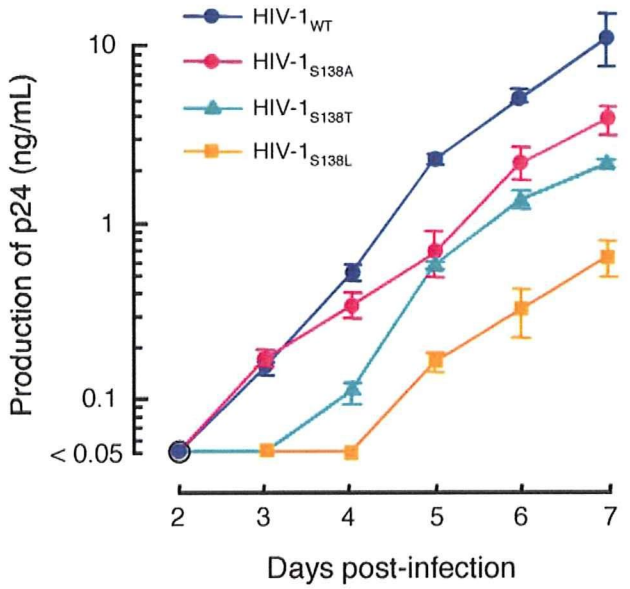

FIGURE 4. **Replication kinetics of HIV-1_{S138X} variants (*X*, any natural amino acid).** HIV-1_{S138A} (*bright red circles*) showed replication kinetics comparable with those seen for HIV-1_{WT} (*blue circles*). Replication of HIV-1_{S138T} (*emerald green triangles*) was reduced, somewhat surprisingly, as both threonine and serine are β-hydroxy amino acids, albeit with different hydrophobicity and torsional flexibility. HIV-1_{S138L} (*orange squares*) also showed reduced replication kinetics. Note that HIV-1_{S138P} and HIV-1_{S138W} failed to replicate (data not shown). Results shown are representative of three independent experiments. An identical order of replication kinetics was observed. Productions of p24 antigen on days 4–7 between HIV-1_{WT} and HIV-1_{S138A} were significant (*t* test, $p < 0.05$).

Structure Modeling—The side chain of amino acid 138 (Ser or Ala) closely contacts with the hydrophobic pocket formed by Leu-44 and Leu-45 in the N-HR. The mutation from Ser to Ala increases hydrophobicity and may help to stabilize the N-HR/C-HR complex related with the potency of the HIV-1 fusion inhibitors (Fig. 5). Larger hydrophobic substitutions such as S138W, S138L, or S138I are likely to sterically interfere with efficient packing of the N-HR and C-HR helices. Similarly, introduction of charged residues at this region of the interface would also disrupt the hydrophobic environment and result in destabilized helix bundles, consistent with the biochemical and virological findings (Figs. 2–4 and Table 1).

Based on crystallographic studies (27, 28), we observe that the T-20 resistance N43D mutation should affect interactions between helices in the 6-helix bundle. Specifically, residue 46 of N-HR is proximal to residue Glu-137 of the C-HR helix of another molecule in the 6-helix bundle. We believe that this increase in proximal negative charges and juxtaposition of Asp-36 next to Glu-137 may destabilize the formation of the 6-helix bundle in a way that results in reduced efficiency of fusion and reduced replication kinetics. Increase of the hydrophobic interactions by introduction of the S138A mutation should help overcome the negative effects of the N43D mutation.

DISCUSSION

In this study we demonstrate that by introducing a secondary resistance mutation into the sequence of peptide-fusion inhibitors such as C34 and T-20, we can suppress efficiently replication of wild-type and of fusion inhibitor-resistant HIV-1. Our circular dichroism analysis revealed that C-HR-based fusion inhibitors that carry secondary resistance mutations can form tight 6-helix bundles with N-HR that contains primary resistance mutations responsible for T-20 resistance. A similar approach has been applied for the development of short hairpin RNA (shRNA) sequences that inhibit HIV-1 replication (29). The synthesized shRNA with mutations that confers resistance to the parental shRNA effectively suppressed replications of shRNA resistant HIV-1 but not wild-type HIV-1. Therefore, it is possible to gain valuable insights from the resistance information and directly apply it to design new peptides or oligonucleotides in the case of shRNA that preempt the viral escape mechanism and suppress resistant variants. Moreover, this strategy should not result in more adverse effect than those that might be obtained during use of the original peptide or oligonucleotide reagents.

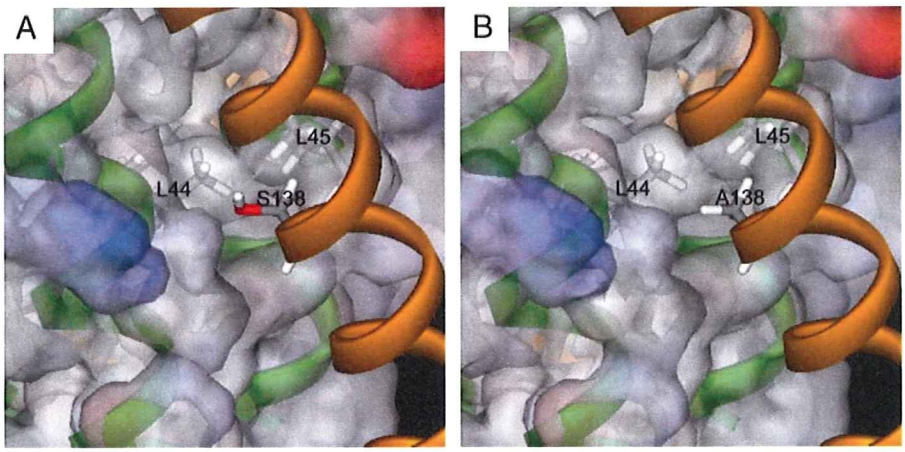

FIGURE 5. **Structure of gp41 at the region near position 138 in the C-HR.** *A*, crystal structure of the N36/C34 complex (PDB code 1AIK). *B*, computational structure modeling of the S138A mutant (N36/$C34_{S138A}$ complex). N-HR and C-HR helices are colored *green* and *orange*, respectively. The van der Waals surface of only N-HR is shown and colored according to the electrostatic potential.

Recently we (6, 30, 31) and others (5) reported that hydrophilic amino

acid substitutions stabilized the α-helix of C-HR peptides and increased their binding affinity to N-HR, thus providing potent anti-HIV activity. This property may be one of the key attributes of the recently developed potent peptide inhibitors, SC34EK (6, 30), T-20EK (31), or T-2429 (5), that have been reported to efficiently inhibit T-20 resistant variants. However, the S138A substitution on T-20 in the present study had little effect on the random coil structure, as judged by CD (data not shown), indicating that $T\text{-}20_{S138A}$ increases its binding affinity not by simply enhancing the α-helicity of this region (5, 6). Our approach of introducing substitutions selected on the basis of the mutation(s) that appears in resistant viruses significantly improved the affinity with N-HR. This approach may complement the effects of enhancing helical stability and may help generate more potent and effective fusion inhibitors for resistant HIV-1 variants.

Other methods have also been employed to improve the potency of HIV fusion inhibitors. For example, T-1249 is a peptide that is based on the T-20 sequence and has improved binding properties (32, 33). It contains 17 changes compared with T-20 (3 additional residues and 14 substitutions to increase the α-helicity/binding affinity according to amino acid sequences of HIV-2 and simian immunodeficiency virus). T-2635 is another efficient peptide fusion inhibitor that was recently developed and is also modified extensively (19 substitutions in 38 amino acids) (5). Also, SC34EK is an electrostatically constrained peptide that also suppresses replication of T-20-resistant variants, and it required 12 substitutions in the original C34 inhibitor (6, 30). Hence, it is possible to improve the potency of existing peptide inhibitors through intense modeling and iterative testing in *in vitro* studies that could lead to the design and synthesis of improved peptide drugs. However, the approach we followed in the design of the $T\text{-}20_{S138A}$ inhibitor is considerably simpler and involves a smaller number of sequence changes (1 residue changed, compared with 19 and 12 in the cases of T-2635 and SC34EK, respectively; see above). It takes advantage of information obtained from the viral evolution under drug pressure and uses the resistance information to design improved inhibitors. In addition, we believe that this approach may be applicable to other targets even when the interactions do not involve helical bundles or detailed information on related systems is not available. Importantly, whenever possible, a combination of the two approaches would likely generate even more effective peptide inhibitors that can suppress replication of resistant variants.

α-Helical structure is a significant factor not only in HIV-1 fusion but also in other examples of protein-protein interactions. Peptide-based drugs have to overcome multiple obstacles, including poor oral bioavailability, less permeability into the target cells, and high cost. Several modifications, such as using arginine-rich peptide tags (34, 35), and chemical treatments (36) have been used to overcome the cell permeability problem. At any rate, peptide-based reagents can be an important tool in the discovery and validation of novel therapeutic targets through *in vitro* experiments. For example, it has been shown that the function of a target protein can be inhibited by designing synthetic peptides that have the amino acid sequence of a domain which is important for the protein function. In such cases the peptides may act as decoys that have antagonistic/agonistic or competitive effects, leading to inhibition of the protein function. Similarly, screening through peptide sequences of proteins may be useful for the identification of functionally important domains that could become future targets for peptide-based or small molecule-based drug development.

In this study we designed peptides tailored to suppress T-20-resistant HIV-1 strains. To our knowledge, this is the first report of direct application of resistance information in drug design and may be applicable to other, unrelated systems. For example, a BH3 domain of the anti-apoptotic protein Bcl-2 has been targeted by an α-helical domain mimic peptide (37, 38). The resulting hydrocarbon-stapled peptide, $SAHB_{A,}$ penetrates into cells via endocytosis pathway and inhibits the function of Bcl-2, inducing apoptosis in transplanted leukemia cells in mice. However, during prolonged therapy with such peptides, leukemic cells could develop resistance to the peptides through substitutions in the Bcl-2 region in the selection process for survival reminiscent of HIV-1. One can envision that our strategy of using mutational resistance information to overcome drug resistance might help in the design of substituted peptides that suppress the resistant variants more efficiently, thus contributing to broader applications of successful peptide-based therapies.

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SC29EK, a Peptide Fusion Inhibitor with Enhanced α-Helicity, Inhibits Replication of Human Immunodeficiency Virus Type 1 Mutants Resistant to Enfuvirtide[▽]

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Peptides derived from the α-helical domains of human immunodeficiency virus (HIV) type 1 (HIV-1) gp41 inhibit HIV-1 fusion to the cell membrane. Enfuvirtide (T-20) is a peptide-based drug that targets the step of HIV fusion, and as such, it effectively suppresses the replication of HIV-1 strains that are either wild type or resistant to multiple reverse transcriptase and/or protease inhibitors. However, HIV-1 variants with T-20 resistance have emerged; therefore, the development of new and potent inhibitors is urgently needed. We have developed a novel HIV fusion inhibitor, SC34EK, which is a gp41-derived 34-amino-acid peptide with glutamate (E) and lysine (K) substitutions on its solvent-accessible site that stabilize its α-helicity. Importantly, SC34EK effectively inhibits the replication of T-20-resistant HIV-1 strains as well as wild-type HIV-1. In this report, we introduce SC29EK, a 29-amino-acid peptide that is a shorter variant of SC34EK. SC29EK blocked the replication of T-20-resistant HIV-1 strains and maintained antiviral activity even in the presence of high serum concentrations (up to 50%). Circular dichroism analysis revealed that the α-helicity of SC29EK was well maintained, while that of the parental peptide, C29, which showed moderate and reduced inhibition of wild-type and T-20-resistant HIV-1 strains, was lower. Our results show that the α-helicity in a peptide-based fusion inhibitor is a key factor for activity and enables the design of short peptide inhibitors with improved pharmacological properties.

The envelope proteins of human immunodeficiency virus (HIV) type 1 (HIV-1) exist as functional trimeric complexes of gp120-gp41 heterodimers and play an important role in viral entry into host cells. Interactions of gp120 with CD4 molecules expressed on the cell surface cause structural changes that allow further interactions with the CXCR4 or CCR5 coreceptor. These interactions also induce a conformational change in gp120 that initiates gp41-mediated membrane fusion that leads to viral entry (4). In the process of fusion, the amino-terminal heptad repeat (N-HR) of gp41 trimer interacts with the carboxyl-terminal heptad repeat (C-HR) of gp41 trimer to form a six-helix bundle that makes viral and cell membranes accessible (3).

Peptides derived from N-HR or C-HR, such as N36 (3, 18) and enfuvirtide (T-20) (30), suppress the six-helix bundle formation, resulting in the inhibition of membrane fusion. T-20 blocks the entry of various HIV-1 strains, even those resistant to inhibitors of reverse transcriptase and/or protease (15, 16). However, T-20-resistant HIV-1 variants, which frequently show mutations in gp41, such as V38A and N43D, have emerged (14, 25, 26, 28, 32). Therefore, novel fusion inhibitors that suppress the replication of T-20-resistant variants are urgently needed.

C34, a C-HR-derived peptide (Fig. 1A), also inhibits fusion in vitro and does so much more efficiently than T-20 (3, 18, 22). Previously, we remodeled C34 by introducing amino acid substitutions that resulted in highly soluble and active derivatives (24). We replaced amino acids at the solvent-accessible site of the helical bundle with glutamate (E) and lysine (K) and maintained those at the interactive site, as these are critical for the interaction with N-HR. In an α-helical heptad repeat, residues separated by three positions (position i versus position $i + 4$) are located on the same side of the helix and are closely positioned in space (Fig. 1B). Hence, we introduced consecutive EK motifs separated by three residues (E at positions i and $i + 1$ and K at positions $i + 4$ and $i + 5$) of the solvent-accessible site of C34, which resulted in a repeat of the following type: X-EE-XX-KK (where X indicates the original amino acid in HIV-1). A C34 derivative, SC34EK, which has two complete and three incomplete X-EE-XX-KK motifs (Fig. 1), showed enhanced anti-HIV-1 activity compared with that of the parental peptide, C34 (24). A similar result was obtained with T-20EK, the peptide derived by introducing this motif into T-20 (23). Circular dichroism (CD) analysis revealed that both the α-helicity of SC34EK and the thermal stability of the N36-SC34EK complex were enhanced. Interestingly, the antiviral activity of SC35EK, which has five complete X-EE-X X-KK motifs, was comparable to that of SC34EK (24), indicating that five complete X-EE-XX-KK motif repeats are not

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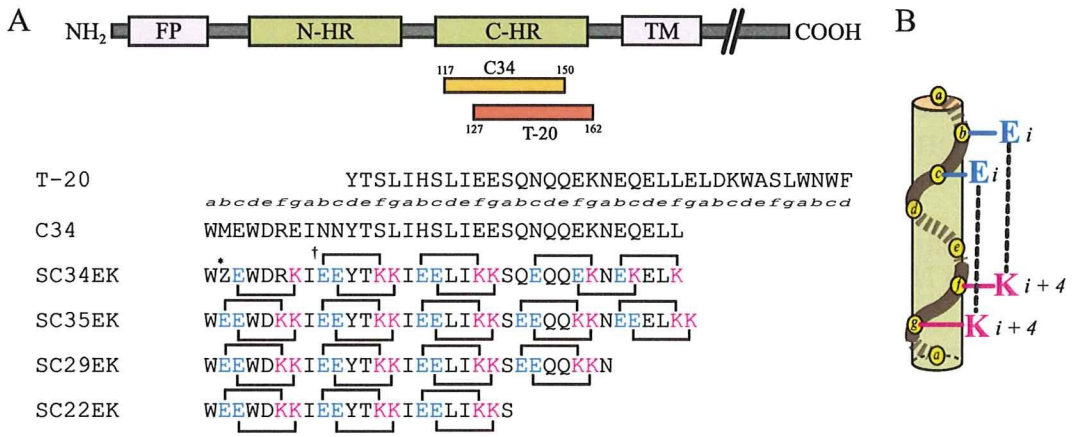

FIG. 1. (A) Schematic diagram of HIV-1 gp41 and sequences of C-HR-derived peptides. FP, fusion peptide; TM, transmembrane domain. The residues at each position in the helical turns are denoted in italics. *Z, an artificial amino acid, norleucine, instead of methionine, to avoid oxidation of the side chain of methionine; †, possible electrostatic interactions indicated by lines and correlating amino acids. (B) One heptad helical turn.

required for strong anti-HIV-1 activity. To address how many complete X-EE-XX-KK motifs are involved in the potent antiviral activity of SC34EK, we synthesized SC29EK and SC22EK (Fig. 1), which contain four and three complete repeats of X-EE-XX-KK, respectively, and evaluated them for their activities against T-20-resistant viruses.

MATERIALS AND METHODS

Cells. HeLa CD4/LTR–β-galactosidase cells obtained from M. Emerman through the AIDS Research and Reference Reagent Program (Germantown, MD) and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G, and 50 μg/ml streptomycin.

Viruses. An HIV-1 infectious clone, pNL4-3 (1), was used for the construction and production of HIV-1 clones. Clones with a certain resistance mutation(s) were introduced by site-directed mutagenesis (29) into the pNL4-3 construct. Although the vast majority of HIV-1 strains have a glycine (G) at position 36 in gp41, the NL4-3 strain used in this study has an aspartic acid (D) residue, which results in impairment of the fusion kinetics of HIV-1 (13, 17). Therefore, in this study, we first constructed a D36G clone, pNL4-3_{D36G}, and used this as a template for the introduction of T-20-resistant mutations, as described previously (21, 31). We constructed three T-20-resistant clones, HIV-$1_{D36G/V38A}$, HIV-$1_{D36G/N43D}$, and HIV-$1_{D36G/N43D/S138A}$ (8), and two C34-resistant clones, HIV-$1_{D36G/N126K}$ and HIV-$1_{\Delta V4/D36G/I37K/N126K/L204I}$ (ΔV4 indicates a five-amino acid [FNSTW] deletion in the V4 region of gp120) (22). Infectious HIV-1 clones were generated by transfection of plasmid clones into 293T cells.

Antiviral agents. Peptide-based fusion inhibitors, including T-20, were synthesized by standard 9-fluorenylmethoxy carbonyl-based solid-phase techniques (24). High-pressure liquid chromatography purification of crude materials on a preparative Cosmosil 5C18 AR-II column with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid gave the desired peptide samples for biological tests. 2′,3′-Dideoxycytidine (ddC) was purchased from Sigma-Aldrich (St. Louis, MO).

Determination of efficacies of antiviral agents. The efficacies of the antiviral agents were determined by multinuclear activation of galactosidase indicator (MAGI) assays (12, 22). Briefly, 10^4 HeLa CD4/LTR-β-galactosidase cells per well were plated in flat 96-well culture plates. On the following day, the cells were inoculated with the HIV-1 clones (60 MAGI U/well, which gave 60 blue cells after 48 h of incubation) and were cultured in the presence of various concentrations of drugs in fresh medium. After incubation for 48 h after virus inoculation, all of the blue cells that were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside in each well were counted. The activities of the test compounds were determined as the concentration that blocked HIV-1 replication by 50% (the 50% effective concentration [EC_{50}]).

Effect of sera on anti-HIV activity. The effect of the FCS concentration on antiviral activity was measured by MAGI assays with FCS at several concentrations (5, 10, 20, and 50%). The effect of serum components on antiviral activity was assessed by MAGI assays. Briefly, T-20 or SC29EK was dissolved in phosphate-buffered saline (PBS), FCS, or serum freshly prepared from HIV-seronegative healthy volunteers at 4 μM; and the mixtures were incubated for 2 h at 37°C. The mixture was diluted to a concentrations of about 1× or 5× the EC_{50} by using a DMEM-based complete medium supplemented with 10% FCS and was subjected to the MAGI assay.

CD analysis. N36- and C-HR-derived peptide complexes were incubated at 37°C for 30 min (final peptide concentration, 10 μM in PBS). The CD spectra were acquired on a spectropolarimeter (model J-710; Jasco Inc., Tokyo, Japan) at 25°C as the average of eight scans. Thermal stability was assessed by monitoring the change in the CD signal at 222 nm as a function of temperature. Thermal unfolding at intervals of 0.5°C was performed after a 15-min equilibration at the desired temperature and an integration time of 1.0 s. The midpoint of the thermal unfolding transition (melting temperature [T_m]) of each complex was determined from the maximum of the first derivative, with respect to the reciprocal of the temperature, of the $[\theta]_{222}$ values.

RESULTS

Antiviral activities of peptides into which EK is introduced. Because W117, W120, and I124, which are crucial for binding to N-HR (2, 3), are located in the N terminus of C34, we deleted the C-terminal region of SC35EK to produce short peptides. SC29EK, which has four complete X-EE-XX-KK motifs, inhibited HIV-1_{NL4-3} infection at a level comparable to that at which SC34EK did (Table 1). As was observed with SC34EK, SC29EK also maintained an inhibitory effect toward T-20-resistant clones. Although SC34EK blocked the replication of C34-resistant clone HIV-$1_{\Delta V4/D36G/I37K/N126K/L204I}$, SC29EK failed to do so. On the other hand, C29, with a 5-amino-acid deletion from the C terminus of C34, exerted drastically reduced antiviral activity. SC22EK, which consisted of three X-EE-XX-KK motifs, also showed much reduced antiviral activity compared with the activities of SC29EK and SC34EK. A native peptide corresponding to SC22EK, C22, exhibited no activity against HIV-1_{NL4-3} at concentrations up to 10 μM (data not shown). Thus, to inhibit the physiological interaction of N-HR and C-HR, a peptide 22 amino acids in length, without modification, may be insufficient. The D36G substitution enhanced the susceptibility of HIV-1 to T-20 (28) but not to C34 or its derivatives (Table 1). These results suggest that four X-EE-XX-KK motifs are required to maintain

TABLE 1. Activities of HIV-1 gp41-derived peptides against T-20-resistant mutants

Virus	EC_{50}[a] (nM)					
	T-20	SC22EK	C29	SC29EK	C34	SC34EK
$HIV\text{-}1_{NL4\text{-}3}$	15 ± 1 (6.3)	217 ± 41 (0.3)	245 ± 42 (4.7)	2.4 ± 0.1 (1.3)	2.3 ± 0.1 (1.0)	1.6 ± 0.2 (0.7)
$HIV\text{-}1_{D36G}$	2.4 ± 0.6	686 ± 94	52 ± 18	1.9 ± 0.0	2.3 ± 0.6	2.4 ± 1.0
$HIV\text{-}1_{D36G/V38A}$	23 ± 8 (9.6)	289 ± 84 (0.4)	504 ± 193 (9.7)	3.0 ± 0.6 (1.6)	4.4 ± 1.4 (1.9)	2.2 ± 0.4 (0.9)
$HIV\text{-}1_{D36G/N43D}$	49 ± 10 (20)	114 ± 36 (0.2)	>1,000 (>19)	4.1 ± 0.6 (2.2)	7.9 ± 0.9 (3.4)	1.6 ± 0.4 (0.7)
$HIV\text{-}1_{D36G/N43D/S138A}$	84 ± 16 (35)	>1,000 (>1.5)	>1,000 (>19)	3.4 ± 0.9 (1.8)	15 ± 2 (6.4)	1.5 ± 0.3 (0.6)
$HIV\text{-}1_{D36G/N126K}$	3.4 ± 0.6 (1.4)	>1,000 (>1.5)	192 ± 22 (3.7)	2.7 ± 0.1 (1.4)	7.0 ± 2.0 (3.0)	12 ± 1 (5.0)
$HIV\text{-}1_{\Delta V4/D36G/I37K/N126K/L204I}$[b]	390 ± 155 (163)	252 ± 71 (0.4)	>1,000 (>19)	50 ± 11 (26)	171 ± 15 (74)	3.0 ± 0.2 (1.3)

[a] Antiviral activity, shown as the EC_{50}, was determined by the MAGI assay. Each EC_{50} represents the mean ± standard deviation obtained from at least three independent experiments. The values in parentheses indicate relative changes (*n*-fold) in the EC_{50} compared with the EC_{50} in the presence of the D36G substitution.
[b] ΔV4 indicates a 5-amino-acid deletion (FNSTW) in the V4 region of gp120.

the inhibitory effect of the peptides on the membrane fusion of HIV-1 strains resistant to T-20, as well as $HIV\text{-}1_{NL4\text{-}3}$.

α-Helicity of the six-helix bundle. To elucidate the mechanism by which SC29EK exerts strong anti-HIV activity, we performed CD analysis of the N36-SC29EK complex. The CD spectrum for the N36-SC29EK complex revealed an α-helix conformation with a characteristic double minimum at 208 nm and 222 nm, similar to the conformations of the N36-C34 and N36-SC35EK complexes. The N36-C29 complex showed an α-helical conformation, while a complex of N36 with C22 showed decreased α-helical spectra (Fig. 2A), in direct correlation to moderately and severely decreased antiviral activities of C29 and C22, respectively. The CD spectra of complexes of N36 peptides containing T-20 resistance-associated mutations with SC29EK, $N36_{V38A}$-SC29EK and $N36_{N43D}$-SC29EK, were almost identical to the CD spectrum of N36 with SC29EK, indicating that SC29EK retains binding affinity for the mutated N36 peptides (Fig. 2B). On the other hand, the mutated N36 peptides and C29 complexes showed little α-helical conformation. These results indicate that introduction of the X-EE-X X-KK motif increases the binding affinity of SC29EK for the mutated N36 peptides.

The thermal stabilities of these complexes were assessed by monitoring the shift in $[\theta]_{222}$ (Fig. 2C). A relatively low T_m (48.5°C) (Fig. 2D) and approximately 80% α-helicity at 37°C (Fig. 2C) were observed with the N36-C29 complex, consistent with its moderate antiviral activity (Table 1). The T_ms of N36- and C-HR-derived peptides into which a X-EE-XX-KK motif was introduced were higher than the T_m of the N36-C34 complex (Fig. 2D), while the T_ms of peptides with the native se-

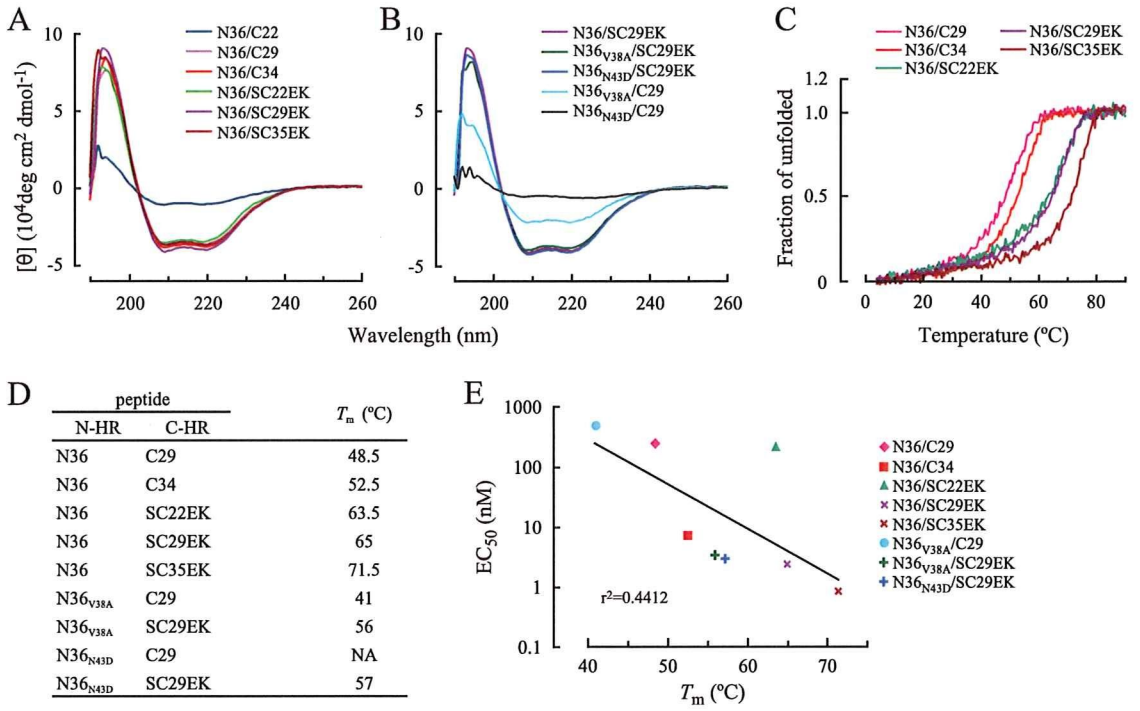

FIG. 2. Analysis of N36 and various C-HR-derived peptides complexes by CD spectroscopy. CD spectra for N36- and C-HR-derived peptide complexes (A) and mutated N36-C29 or SC29EK complexes (B). (C) Temperature-dependent transitions of the dissociation degree of N36 and various C-HR-derived peptide complexes. (D) T_ms of complexes of various N-HR peptides and C-HR peptides. NA, not available. (E) Relation between EC_{50}s of C-HR-derived peptides and T_ms of N36 and various C-HR-derived peptide complexes. The strength of the correlation between EC_{50}s and T_ms is increased (r^2 = 0.8002) when the data for SC22EK are excluded.

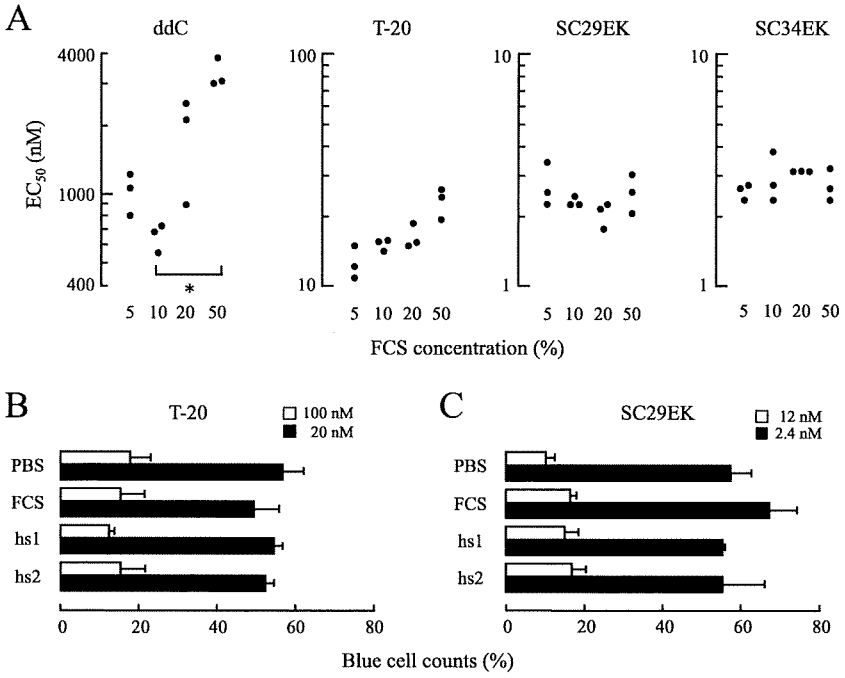

FIG. 3. Effect of serum components on antiviral activity. Antiviral activities in the presence of serum at various concentrations (5, 10, 20, and 50%) were determined by the MAGI assay. (A) Black dots indicate EC_{50}s (nM), each of which was determined three times independently (*, $P = 0.01$ by Student's t test). Antiviral activities of T-20 (B) and SC29EK (C) in human HIV-seronegative sera (hs1 and hs2) were assessed by counting the number of blue cells. Bars indicate the percentages of blue cell counts in PBS, FCS, and human serum compared with the count obtained with no antiviral agents (control, for which the value was 100%). Error bars represent the standard deviation of each mean.

quence but without the introduced motif were lower. The relationships between the EC_{50}s of C-HR-derived peptides and their T_ms are shown in Fig. 2E. The correlation between the EC_{50} and the T_ms was weak ($r^2 = 0.4412$); however, with the exclusion of the data for SC22EK, which showed weak antiviral activity, despite its high T_m, the strength of this correlation was increased ($r^2 = 0.8002$), suggesting that other factors, including solubility and intrapeptide interactions, may be involved in the enhanced antiviral activity of EK-containing peptides.

Effect of serum on antiviral activity. Finally, we assessed the anti-HIV activity of SC29EK in the presence of a high concentration of FCS and in fresh human serum. The activities against HIV-1_{NL4-3} in the presence of various concentrations of FCS (5, 10, 20, and 50%) were determined. A reverse transcriptase inhibitor, ddC, was used as a control. The antiviral activity of ddC was decreased in a concentration-dependent manner (Fig. 3A). In the presence of 50% FCS, the reduction in the EC_{50} of ddC was statistically significant ($P = 0.01$). Similarly, but to a much lesser extent, the EC_{50} of T-20 appeared to be reduced with the FCS concentration in a concentration-dependent manner. Even in the presence of 50% FCS, the mean EC_{50} was comparable to the EC_{50}s in 5% and 10% FCS ($P = 0.082$ and 0.075, respectively). However, the effects of SC29EK and SC34EK were less affected by increased FCS concentrations.

For further evaluation, sera freshly isolated from two HIV-seronegative healthy volunteers were prepared. T-20 and SC29EK incubated for 2 h at 37°C in fresh human serum, FCS, or PBS were diluted with a DMEM-based complete medium supplemented with 10% FCS and were subjected to the MAGI assay. The final FCS concentrations in the various sera that included FCS in these diluted mixtures ranged from 9.75 to 12.2%. Because the antiviral activities of T-20 and SC29EK were not significantly influenced by the FCS concentration (Fig. 3A), it is unlikely that the differences in the FCS concentrations in this experiment had any effect on their antiviral activities. Compared with the inhibitory effects of the PBS-treated peptides, small changes in the inhibitory effects of both T-20 and SC29EK treated with FCS and human sera were observed (Fig. 3B and C). Taken together, these findings suggest that SC29EK stably exerts its strong anti-HIV-1 activity in vivo in the same manner that T-20 does.

DISCUSSION

We show here that SC29EK inhibits the membrane fusion of T-20-resistant HIV-1 strains, suggesting that four X-EE-X X-KK motifs are sufficient to inhibit the fusion of T-20-resistant variants. As revealed by the EC_{50}s and T_ms (Table 1 and Fig. 2), resistance-associated mutations in the N-HR region, such as V38A and N43D, seem to decrease the binding affinity of C-HR-derived peptides for N-HR. Therefore, HIV-1 strains with V38A or N43D show resistance to T-20. However, the anti-HIV-1 activity of SC29EK was less affected by these mutations, because at the physiological temperature for HIV-1 replication, SC29EK showed a stable interaction with N36 peptides containing mutations conferring resistance to T-20. The activity of SC29EK against the C34-resistant clone HIV-$1_{\Delta V4/D36G/I37K/N126K/L204I}$ was decreased, while SC34EK maintained its activity. One of the primary mutations underlying C34 resistance, I37K, is located close to but outside of the

putative binding site of SC29EK. Previously, we reported that an N126K substitution in C-HR enhances the intra-gp41 binding of N-HR and C-HR (22); therefore, we hypothesized that the activity of SC29EK might be decreased by competition with C-HR with the N126K mutation. However, SC29EK also inhibits the entry of HIV-$1_{D36G/N126K}$. Although no structural analysis of the mutated six-helix bundle was performed, it is possible that mutations conferring C34 resistance might induce some structural changes at or adjacent to the SC29EK binding site, because a peptide shortened by a further 7 amino acids, SC22EK, suppressed the entry of the C34-resistant clone.

C34 itself did not have an α-helical spectrum, while SC29EK did (data not shown). SC29EK may achieve its strong antiviral activity by forming an α-helix as a result of E/K substitutions on the solvent-accessible site (Fig. 1). CD analysis shows that HIV-1 builds up resistance to T-20 by introducing certain mutations in N-HR, such as V38A and N43D, which reduce the binding affinity between N-HR and C-HR. SC29EK can efficiently inhibit the fusion of these mutant HIV-1 strains, suggesting that the ability of SC29EK to bind to mutated N-HR and its weak affinity for C-HR are maintained. On the other hand, the D36G, N126K, and S138A mutations increase viral fusion activity (13) by enhancement of the binding affinity of C-HR for N-HR (22, 31). SC29EK effectively suppresses the replication of viruses that have these mutations, such as HIV-1_{D36G}, HIV-$1_{D36G/N43D/S138A}$, and HIV-$1_{D36G/N126K}$. This indicates that the binding capacity of SC29EK is stronger than that of mutated C-HR containing the N126K or the S138A mutation. Therefore, the monomeric α-helical form may inhibit the interactions of N-HR and C-HR with mutations that affect their binding affinity and thus the formation of the six-helix bundle.

Although SC22EK has enhanced α-helicity and a high T_m, it has less antiviral activity than SC29EK. In the interaction between N-HR- and C-HR-derived peptides, while the cavity-forming region (from L54 to Q66) of the C terminus of N-HR (the "pocket") and the cavity-binding region (side chains of W117, W120, and I124) of the N terminus of C-HR (the "knob") play an important role (2, 3, 10), another region of C-HR may also be required. A constrained 14-residue peptide (C14linkmid), which corresponds to the knob region, shows chemical cross-linking and contains amino acid substitutions (27), and it is about 15,000-fold less active than SC29EK, which contains proximal regions in addition to the knob region. These findings also suggest that the knob region of C-HR is important but not sufficient for the formation of a stable complex. Another possible explanation of the weak activity of C14linkmid is that because not only the binding of N-HR and C-HR but also dynamic structural changes are easily anticipated during fusion, it would be difficult to maintain tight binding to the target N-HR due to its rigid constrained form. To maintain the binding of C-HR to N-HR despite such drastic conformational changes during fusion, there may be some unknown interaction, besides the interaction between the pocket and the knob regions, that is necessary for membrane fusion. At present, we cannot conclude whether (i) the length of the peptide itself is crucial, (ii) some other domain has a role, or (iii) a combination of both is important. Further experiments will be needed to clarify the mechanism of inhibition. Such information will be valuable for the generation of effective short peptide inhibitors or small molecules. To generate effective small-molecule inhibitors, if the second possibility is correct, a combination of two agents, one of which interacts with the pocket and the other of which interacts with an unidentified domain, should provide enhanced efficacy. To date, only a limited number of small-molecule compounds that inhibit the six-helix bundle formation with marginal activities have been reported (5, 9, 11), although among the peptide-based inhibitors, several effective peptides have been developed, including T1249 (7), SC34EK (24), T2635 (6), and T-20EK (23).

The T_m of the N36-SC29EK complex was higher than that of the N36-C29 complex, suggesting that EK substitutions reinforced the affinity of binding to N-HR through enhanced α-helicity. It has been considered that the enhanced α-helical structure is maintained by intrahelical salt bridges formed by the introduction of EK substitutions (19). We recently revealed that an electrostatic interaction formed by the EK alignment is involved in enhanced α-helicity (22a), indicating that the strong α-helical stability of SC29EK is probably provided by a mechanism identical to that for SC34EK. Similar peptides with substitutions of glutamate and arginine provided to increase α-helicity have been reported (6). These peptides also increase the stability of the helix and have activity against T-20-resistant HIV-1. Moreover, these peptides were relatively stable in an in vivo model. It is possible that enhanced binding affinity confers nonspecific binding to other α-helical regions of cellular proteins, for example, human serum albumin, which contains 31 α-helical regions (20). However, this effect will be minimal, because the antiviral activity of SC29EK was highly stable in the presence of higher concentrations of FCS and was less affected by human serum.

In this study, we demonstrated that a 29-amino-acid short peptide, SC29EK, suppresses the replication of T-20-resistant variants. SC29EK maintained its activity in the presence of high concentrations of sera, indicating that SC29EK is a candidate short peptide fusion inhibitor.

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Synonymous mutations in stem-loop III of Rev responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide

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ABSTRACT

Primary mutations in HIV-1 that are directly involved in the resistance to enfuvirtide have been well documented. However, secondary mutations that are associated with primary mutations and contribute little to the resistance still remain to be elucidated. This study reveals that synonymous mutations at gp41 Q41 (CAG to CAA) or L44 (UUG to CUG) act as secondary mutations. Complementary mutations in the nucleotide level are located in the Rev responsive element (RRE) of the HIV-1 RNA-genome and maintain the replication kinetics of HIV-1 through increasing the structural stability of stem-loop III in the RRE. Therefore, synonymous mutations in the gp41/RRE sequence improve the viral replication impaired by the primary mutations and play a key role as secondary (complementary) mutations.

1. Introduction

Enfuvirtide (T-20), an HIV-1 fusion inhibitor which has been approved for the treatment of HIV-1 infected patients, successfully suppresses the replication of HIV-1 even in strains resistant to various reverse transcriptase and protease inhibitors (Fung and Guo, 2004; Lalezari et al., 2003; Lazzarin et al., 2003; Manfredi and Sabbatani, 2006). However, HIV-1 variants resistant to T-20 have emerged after prolonged T-20 therapy (Lu et al., 2006; Marcelin et al., 2004; Sista et al., 2004; Wei et al., 2002). The majority of resistant variants develop primary mutations to T-20, including V38A and N43D, in the consensus sequence, GIVQQQNNLL (DIVQQQNNLL for NL4-3) motif of the gp41 within amino acid positions 36–45 (Cabrera et al., 2006; Fikkert et al., 2002; Lu et al., 2006; Marcelin et al., 2004; Menzo et al., 2004; Mink et al., 2005; Poveda et al., 2002; Sista et al., 2004; Su et al., 2006; Wei et al., 2002). Few studies have so far addressed secondary mutations for resistance to T-20.

Primary and secondary mutations were recently well-defined for C34, a peptide derived from the gp41 carboxyl terminus heptad repeat (C-HR) in vitro (Nameki et al., 2005). A30V and D36G in the gp41 act as secondary mutations which enhance the replication kinetics impaired by primary mutations. Interestingly, these mutations are complementarily located in stem-loop IIA and C (Fig. 1) of the Rev responsive element (RRE) which is an essential RNA structure for transporting non- and singly spliced viral RNA to the cytoplasm from the nucleus (Olsen et al., 1990; Zapp and Green, 1989). Most recently, T18A and V38A complementarily located in the stem IIA of the RRE has been detected in clinical isolates treated with T-20 (Svicher et al., 2008). Co-presence of T18A and V38A is associated with high level of viral load. These data support our recent in vitro results that these secondary mutations in stem II maintain HIV-1 replication through substitutions at the nucleotide as well as the amino acid level (Nameki et al., 2005). This study analyzed the nucleotide sequences of T-20 resistant variants deposited in the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) and revealed that synonymous mutations in stem-loop III of the RRE maintain HIV-1 replication impaired by N43D, one of primary mutations for T-20 resistance.

2. Materials and methods

2.1. Antiviral agents and cells

T-20 was synthesized as described previously (Otaka et al., 2002). 2′,3′-Dideoxycytidine (ddC) was purchased from Sigma (St.

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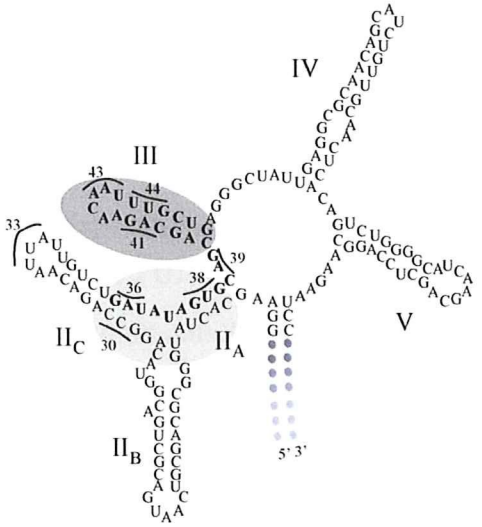
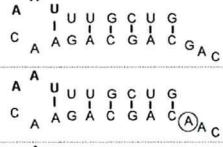
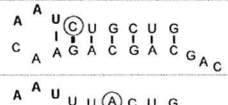
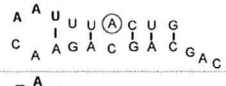
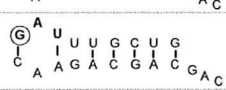
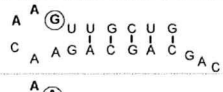
(A)



(B)

Mutations: Amino acid	Mutations: Nucleotide	Secondary structure of stem loop III	ΔG (kcal/mol)
None (WT: NL4-3)	None		- 6.5
Q39Q	CAG → CA**A**		- 6.8
Q41Q	CAG → CA**A**		- 6.4
L44L	UUG → **C**UG		- 9.1
L44L	UUG → **UU**A		0.1
N43D	AAU → **G**AU		- 6.5
N43D L44L	AAU → **G**AU UUG → **C**UG		- 9.0
Q39Q N43D	CAG → CA**A** AAU → **G**AU		- 6.8
Q41Q N43D	CAG → CA**A** AAU → **G**AU		- 6.4
N43K	AAU → AA**A**		- 4.9
N43K	AAU → AA**G**		- 5.4
N43K L44L	AAU → AA**A** UUG → **C**UG		- 7.7
N43K L44L	AAU → AA**G** UUG → **C**UG		- 7.9
Q40H L45M	CAG → CA**T** CUG → **A**UG		- 4.5

(C)

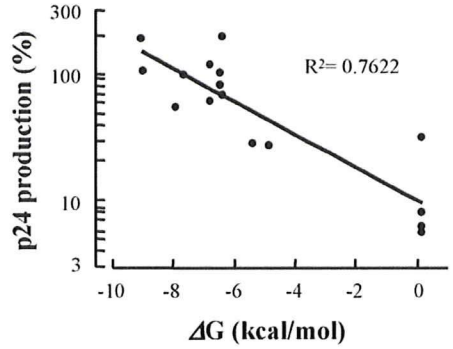


Fig. 1. (A) Secondary structure of RRE depicted based on references (Olsen et al., 1990; Zapp and Green, 1989). T-20-resistant mutations are accumulated between amino acid position from 36 to 45 of gp41 and nucleotides coding for these amino acids, are given in bold. Stem-loop II (light gray region) was examined as described previously (Nameki et al., 2005) and stem-loop III (dark gray region) was analyzed in this study. (B) Amino acid substitutions in the gp41 with their nucleotide changes and putative secondary structures and their stabilities of stem-loop III are shown. Nucleotide substitutions are indicated in bold and circle. Newly generated nucleotide pairs are indicated by the bold line. The stability of stem-loop III defined by ΔG value was calculated using the MFold program version 3.2 (Mathews et al., 1999; Zuker, 2003). (C) Correlation of the amounts of p24 production and stability of stem-loop III (ΔG value) is shown. Plots of Q41H and L45M were included (see text) but that of V38A was excluded since V38A is located in stem IIA. Since replication of N43D containing clones were extremely low, p24 productions obtained from D36G combined N43D or K containing clones, were used. When excluding data of D36G combined with N43D or N43K and including that of N43K without D36G, correlation coefficient (R^2) was 0.72.

Louis, MO). HeLa-CD4-LTR-β-gal cells were used for the drug susceptibility assay (MAGI assay) as described (Nameki et al., 2005). MT-2 and 293T cells were grown in RPMI1640- and Dulbecco's modified Eagle's medium (DMEM)-based culture medium, respectively.

2.2. Generation of recombinant viruses

An HIV-1 infectious clone, pNL4-3, which was kindly provided by Dr. H. Sakai, Institute for Virus Research, Kyoto University (Kyoto, Japan), was used for the construction and the production of HIV-1 variants. To generate major T-20-resistent molecular clones, desired mutations were introduced into the Nhe I-BamH I region (1221 bp) of pSLgp41WT, which encoded nucleotides 7250–8470 of pNL4-3, by site-directed mutagenesis as described (Nameki et al., 2005). The Nhe I-BamH I fragments were then inserted into pNL4-3, generating various molecular clones with the desired mutations. Viruses were recovered from the supernatant of the transfected 293T cells and stored at −80 °C until use. A wild type HIV-1, HIV-1WT, was generated by transfection of pNL4-3 into 293T cells.

2.3. Determination of titer and drug susceptibility of HIV-1

The titer and peptide-sensitivity of infectious clones was determined by the MAGI assay with some modifications (Kimpton and Emerman, 1992; Kodama et al., 2001; Maeda et al., 1998). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in 96-well flat microtiter culture plates. On the following day, the cells were inoculated with the supernatant of 293T cells transfected with HIV-1 clones and the titrated HIV-1 clones in the presence of various concentrations of drugs in fresh medium, for determination