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. 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 induced 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)3 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 a-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 a-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

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 the parental peptides. These new changes enhance the antiviral potency against resistant variants. Hence, T-20 with a S138A substitution (T-20S138A), 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 the stability of the N-HR-C-HR complex (19). Similar results were observed for C34 with N126K (C34N126K), also secondary mutations for T-20-resistant variants in vitro (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 original peptide inhibitor. These new changes enhance the antiviral potency against resistant variants. Hence, T-20 with a S138A substitution (T-20S138A), 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 the stability of the N-HR-C-HR complex (19). Similar results were observed for C34 with N126K (C34N126K), also secondary mutations for T-20-resistant variants in vitro (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-1NL4-3), 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-1NL4-3 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 supernatants were 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 (10⁶ 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.
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-1NL4-3 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 C34 (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 passage 24 (P-24), where the concentration of SC34EK 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 C34 (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 SC34EK to the P-45 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.
Acquisition of resistance to SC34EK seems to be faster than that to SC34 (Fig. 2). However, actual EC_{50} 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 the N-L4-3 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 V182I/P203S/L204I/S241F/H258Q/A312T, again conferred moderate resistance to SC34EK and T-20 (4.9- and 18-fold, respectively), but not to C34 and SC34. Substitutions other than A96D and H312Y had little influence on the susceptibility to SC34EK (range of fold-decrease in resistance was 0.6–3.3). However, HIV-1SC34EK(P-120)gp160, which contains multiple substitutions, D36G/Q41R/N43K/A96D/N126K/H132Y/L105F, 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). Ala^{37} 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 H312Y had little influence on the susceptibility to SC34EK (range of fold-decrease in resistance was 0.6–3.3).

We also evaluated the effect of mutations in the gp120 on drug susceptibility. The two clones, HIV-1SC34IP(-123)gp120 and HIV-1SC34EK(P-120)gp120 containing 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, showed no significant resistance to SC34EK.

### Table 1

| Mutations(s) | ddC | T-20 | C34 | SC34 | SC34EK |
|--------------|-----|------|-----|------|--------|
| HIV-1NL4-3 a | 430 ± 121 | 20 ± 3 | 3.3 ± 0.6 | 1.4 ± 0.3 | 0.7 ± 0.4 |

a HIV-1NL4-3 was used as wild-type virus.

### Table 2

| gp41 | gp120 | gp160 |
|------|-------|-------|
| SC34(P-122)gp41 | SC34(P-122)gp120 | SC34(P122)gp41 |
| 339 ± 95 (0.8) | 45 ± 2 (2.3) | 1.727 ± 255 (86) |
| 0.8 ± 0.3 (0.2) | 0.2 ± 0.03 (0.1) | 2.189 ± 287 (1,564) |
| 0.2 ± 0.05 (0.3) | | 33 ± 4 (47) |

5 K. Shimura, D. Nameki, K. Kajiwara, K. Watanabe, Y. Sakagami, S. Oishi, N. Fuji, M. Matsuoka, S. G. Sarafianos, and E. Kodama, unpublished data.
TABLE 2

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

Anti-HIV activity was determined using MAGI assay. Data are shown as mean ± 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-1NL4–3, is shown in parentheses.

| Mutation(s) | ddC | T-20 | C34 | SC34 | SC34EK |
|-------------|-----|------|-----|------|--------|
| HIV-1NL4–3   | 430 ± 121 | 20 ± 3 | 3.3 ± 0.6 | 1.4 ± 0.3 | 0.7 ± 0.4 |
| gp21        |     |      |      |      |        |
| D36G        | 392 ± 93 (0.9) | 1.0 ± 0.1 (0.05) | 8.1 ± 3.4 (2.5) | 0.6 ± 0.2 (0.4) | 0.7 ± 0.2 (1.0) |
| Q41R        | 259 ± 19 (0.6) | ND² | 125 ± 50 (38) | 5.3 ± 0.4 (3.8) | 2.3 ± 0.6 (3.3) |
| N43K        | 220 ± 50 (0.5) | 278 ± 24 (14) | 180 ± 54 (55) | 2.7 ± 0.6 (1.9) | 2.3 ± 0.8 (3.3) |
| A96D        | 255 ± 13 (0.6) | >1,000 (>50) | 12 ± 1.3 (3.6) | 2.8 ± 1.2 (2.0) | 4.4 ± 0.9 (6.3) |
| N126K       | 256 ± 18 (0.6) | 27 ± 5 (1.4) | 8.0 ± 2.9 (2.4) | 1.3 ± 0.4 (0.9) | 0.4 ± 0.1 (0.6) |
| H132Y       | 394 ± 83 (0.9) | 363 ± 70 (18) | 3.6 ± 1.4 (1.1) | 2.7 ± 1.3 (1.9) | 3.4 ± 0.4 (4.9) |
| E151K       | 277 ± 16 (0.6) | ND | 2.3 ± 0.6 (0.7) | 1.1 ± 0.2 (0.8) | 1.4 ± 0.4 (2.0) |
| V182I       | 866 ± 181 (1.6) | 26 ± 8 (1.3) | 4.5 ± 1.2 (1.4) | 1.3 ± 0.3 (0.9) | 0.7 ± 0.2 (1.0) |
| P203S       | ND | ND | ND | ND | ND |
| L204I       | 226 ± 12 (0.5) | 13 ± 4 (0.7) | 3.9 ± 0.1 (1.2) | 1.5 ± 0.3 (1.1) | 0.6 ± 0.1 (0.9) |
| P203S/L204I | 646 ± 208 (1.5) | 23 ± 5 (1.2) | 3.5 ± 0.1 (1.1) | 1.6 ± 0.2 (1.1) | 1.5 ± 0.2 (2.1) |
| S241K       | 420 ± 75 (1.0) | 20 ± 3 (1.0) | 6.3 ± 0.2 (1.9) | 1.6 ± 0.3 (1.1) | 0.6 ± 0.1 (0.9) |
| H258Q       | 381 ± 25 (0.9) | 54 ± 19 (2.7) | 13 ± 3 (3.9) | 1.8 ± 0.4 (1.3) | 0.7 ± 0.4 (1.0) |
| A312T       | ND | ND | ND | ND | ND |
| H258Q/A312T | 633 ± 140 (1.5) | 28 ± 5 (1.4) | 6.0 ± 0.7 (1.8) | 1.3 ± 0.2 (0.9) | 0.7 ± 0.2 (1.0) |
| SC34EK(P-120)gp41¹ | 536 ± 20 (1.2) | 112 ± 38 (5.6) | 70 ± 9 (21) | 20 ± 1 (14) | 75 ± 8 (107) |

gp120

| SC34EK(P-120)gp120² | 399 ± 85 (0.9) | 70 ± 16 (3.5) | 1.4 ± 0.4 (0.4) | 0.2 ± 0.3 (0.1) | 0.3 ± 0.1 (0.4) |

gp160

| SC34EK(P-120)gp160³ | 344 ± 42 (0.8) | 435 ± 139 (22) | 378 ± 133 (115) | 5.2 ± 0.5 (3.7) | 72 ± 18 (103) |

¹ HIV-1NL4–3 was used as wild-type virus.
² ND, not determined.
³ HIV-1SC34EK(P-120)gp41 contains D36G/Q41R/N43K/A96D/N126K/H132Y/V182I/P203S/L204I/S241F/H258Q/A312T mutations in gp41-coding region.
⁴ HIV-1SC34EK(P-120)gp160 contains V37A/V59I/S100K/S115N/R138S/D139N/A310T mutations in gp41-coding region.
⁵ HIV-1SC34EK(P-120)gp120 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.
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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 and 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-1NL4–3 was used as wild-type strain (HIV-1WT). The presented data are mean ± 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.

T-20, C34, SC34, and SC34EK. Instead, they exhibited hyper-sensitivity 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). Taken together, these results and phenotypic analyses (Tables 1 and 2) indicate that SC34- and SC34EK-selected mutations 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-1SC34(P-120)gp41 was partially restored by mutations in the gp120 (HIV-1SC34(P-120)gp120). Similarly, SC34EK-selected mutations in gp120 partially improved the replication capacity of HIV-1SC34EK(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 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-
type N-HR. The mutant peptides, N36SC34res and N36SC34EKres, harboring SC34-selected mutations (D36G/I37K/R46K/Q52R/Q56R) and SC34EK-selected mutations (D36G/Q41R/N43K) were prepared through chemical synthesis (Fig. 4A). C34SC34res and C34SC34EKres 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-helices were observed in either N36SC34res or N36SC34EKres (Fig. 4B). Additionally, few significant temperature-dependent changes of spectra were observed in both N36SC34res and N36SC34EKres 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 unfolding transition ($T_m$) of N36-C34 was 53.3 °C, whereas N36SC34 and N36SC34EK formed more stable complexes ($T_m$ values of 65.4 °C for N36SC34 and 71.4 °C for N36SC34EK; Fig. 4C). The resistance mutations in the C-HR enhanced binding stability; $T_m$ of 64.1 °C for N36C34SC34res and 57.5 °C for N36C34SC34EKres. C34, C34SC34res, and C34SC34EKres maintained binding capacity to both N36SC34res and N36SC34EKres (Fig. 4C). SC34 and SC34EK no longer formed stable complexes with N36SC34res and N36SC34EKres, 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-helical-
Antiviral activity of resistant mutation-introduced N36 (N36SC34, N36SC34EK, and original N36) (4) or C34 (C34SC34, C34SC34EK, and original C34) peptides (B) against HIV-1NL4-3 (blue), HIV-1SC34EK(P-122)gp41 (pink), and HIV-1SC34EK(P-120)gp41 (green) was determined using the MAGI assay. Peptide sequences are shown in Fig. 4A. HIV-1NL4-3 was used as the wild-type virus. HIV-1SC34EK(P-122)gp41 contained D36G/I37K/R46K/Q52R/Q56R/N126K/S138A/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.

**FIGURE 5. Antiviral activity of resistant mutation-introduced peptides.**

Antiviral activity of resistant mutation-introduced N36 (N36SC34, N36SC34EK, and original N36) and C34 (C34SC34, C34SC34EK, and original C34) peptides, respectively (Fig. 4A). HIV-1NL4-3 (A) or C34 (C34SC34, C34SC34EK, and original C34) peptides (B) against HIV-1NL4-3 (blue), HIV-1SC34EK(P-122)gp41 (pink), and HIV-1SC34EK(P-120)gp41 (green) was determined using the MAGI assay. Peptide sequences are shown in Fig. 4A. HIV-1NL4-3 or C34 were used as wild-type viruses. HIV-1SC34EK(P-122)gp41 contained D36G/I37K/R46K/Q52R/Q56R/N126K/S138A/E151K/K154N/N163D/L204I/L210F mutations. HIV-1SC34EK(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.

**TABLE 3**

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

| Inhibitors | HIV-1NL4-3 | HIV-1SC34(P-122)gp41 | HIV-1SC34EK(P-120)gp41 |
|------------|------------|----------------------|-----------------------|
| T-1249     | 0.4 ± 0.07  | 0.5 ± 0.06 (1.3)     | 0.5 ± 0.03 (1.3)      |
| T-2410     | 1.0 ± 0.3   | 3.8 ± 13 (38)        | 3.7 ± 0.6 (37)        |
| T-2429     | 2.2 ± 0.8   | 5.9 ± 13 (27)        | 5.4 ± 1.1 (2.5)       |
| T-2544     | 0.9 ± 0.3   | 1.5 ± 0.5 (1.7)      | 1.4 ± 0.5 (1.6)       |
| T-2635     | 0.3 ± 0.08  | 0.4 ± 0.1 (1.3)      | 1.1 ± 0.2 (3.7)       |
| T-290676   | 0.7 ± 0.2   | 2.9 ± 0.8 (4.1)      | 4.6 ± 0.6 (6.6)       |
| Sifuvirdine| 1.7 ± 0.5   | 340 ± 55 (200)       | 35 ± 8.3 (21)         |
| T-20EK     | 6.4 ± 0.8   | 1.548 ± 90 (242)     | 2.650 ± 261 (414)     |

a HIV-1NL4-3 was used as wild-type virus.

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

c HIV-1SC34EK(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-1NL4-3 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-2410, T-2429, T-2544, T-2635, and T-290676 retained activity against HIV-1SC34(P-122)gp41, whereas T-2410, sifuvirdine, and T-20EK showed a decreased effect to various extents. Similarly, sifuvirdine and T-20EK had reduced activity against HIV-1SC34EK(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...
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 Lys137 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-20S138A and C34N126K 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 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 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 improved 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-1SC34EK(P-120) and HIV-1SC34EK(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-1SC34EK(P-120) and HIV-1SC34EK(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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