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HIV-1 Resistance Mechanism to an Electrostatically Constrained Peptide Fusion Inhibitor That Is Active against T-20-Resistant Strains

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T-20EK is a novel fusion inhibitor designed to have enhanced α-helicity over T-20 (enfuvirtide) through engineered electrostatic interactions between glutamic acid (E) and lysine (K) substitutions. T-20EK efficiently suppresses wild-type and T-20-resistant variants. Here, we selected T-20EK-resistant variants. A combination of L33S and N43K substitutions in gp41 were required for high resistance to T-20EK. While these substitutions also caused resistance to T-20, they did not cause cross-resistance to other known fusion inhibitors.

Enfuvirtide (T-20), a 36-amino-acid peptide derived from the C-terminal heptad repeat (C-HR) of HIV-1 gp41, has been approved as the first fusion inhibitor of HIV-1 entry. T-20 inhibits HIV-1 replication by interfering with the formation of the fusion intermediate six-helix bundle, which is composed of three N-terminal heptad repeats (N-HRs) and three C-HRs arranged in an antiparallel orientation (1). Because of its unique mechanism of action, T-20 effectively suppresses replication of HIV-1 resistant to inhibitors targeting the reverse transcriptase and protease (2, 3). However, long-term therapy with a T-20-containing regimen can result in the emergence of T-20-resistant strains (4, 5). These strains contain substitutions at the N-HR region of gp41, including G36D, V38A, and N43K/D, both *in vitro* and *in vivo*, and exhibit reduced susceptibility to T-20 through decreased binding of T-20 to the mutated N-HR (6–14). To suppress replication of such variants and obtain durable efficacy in HIV-1-infected patients, new fusion inhibitors are needed.

To date, several novel fusion inhibitors have been developed, including tifuvirtide (T-1249) (15), sifuvirtide (SFT) (16), and T-2635 (TRI-1144) (17), that potently suppress replication of T-20-resistant variants (Fig. 1A), as well as D-peptide-based (18) or small-molecule inhibitors (19). We recently developed the electrostatically constrained fusion inhibitors SC35EK and its 29-residue shorter form, SC29EK, which also inhibit replication of T-20-resistant HIV-1 (20, 21). These are peptides with electrostatic interactions between glutamic acid (E) and lysine (K) substitutions placed at the *i* and *i* + 4 positions in the solvent-interacting site (EK motif) and are designed to enhance the α-helicity of the peptides (22). The enhancement in α-helicity correlates well with an enhancement in binding affinity for the targeted region and appears to be a key determinant for inhibition of T-20-resistant HIV-1. In addition to C34 (Fig. 1A), we have also applied the EK modification to T-20, termed T-20EK, that shows sustained activity to T-20-resistant variants and HIV-2 strains (23). Moreover, T-20EK showed activity in an animal model (24). To address the mechanism of HIV-1 resistance to T-20EK *in vitro*, we selected T-20EK-resistant HIV-1 strains by using a dose escalation method, identified the primary substitutions that caused resistance to this inhibitor, and evaluated susceptibility of the T-20EK-resistant strains to other fusion inhibitors.

Selection passages were carried out in MT-2 cells using HIV-1_{NL4-3} as the starting wild-type virus (25, 26). The first HIV-1 mutants with enhanced susceptibility to T-20EK emerged at passage 22 (P-22) and were A314T in gp120 and D36G in gp41 (Fig. 1B). The D36G substitution has been widely observed in HIV-1 strains and is thought to contribute to efficient replication rather than causing resistance by decreasing binding to the inhibitor (10, 27, 28). At P-44, we observed the K63N change and a mixture of asparagine and lysine at residue 43 (N43N/K) in gp41. Substitutions in gp120 (see Fig. S1 in the supplemental material) appear to be polymorphisms, because these substitutions were not directly involved in resistance (see Table S1 in the supplemental material). Moreover, S128N and S162N are reported as polymorphisms in the Los Alamos Database (Los Alamos National Library, HIV Sequence Database; http://www.hiv.lanl.gov) and are observed as mixed viruses over a relatively long period of time. We (21, 25, 26) and others (29) have previously reported that substitutions in gp120 can enhance fusion kinetics (30, 31) but do not significantly affect susceptibility to fusion inhibitors. Finally, HIV-1 acquired L33S, N43K, and cytoplasmic tail (CT) substitutions, resulting in viruses that replicated efficiently even in the presence of 1,000 nM T-20EK.

We prepared HIV-1 recombinant clones with the substitutions discovered during our passages and determined the antiviral activities of T-20EK and other peptides against the T-20EK-resistant variants (Fig. 1B) and clones by using a MAGI (multinuclear ac-

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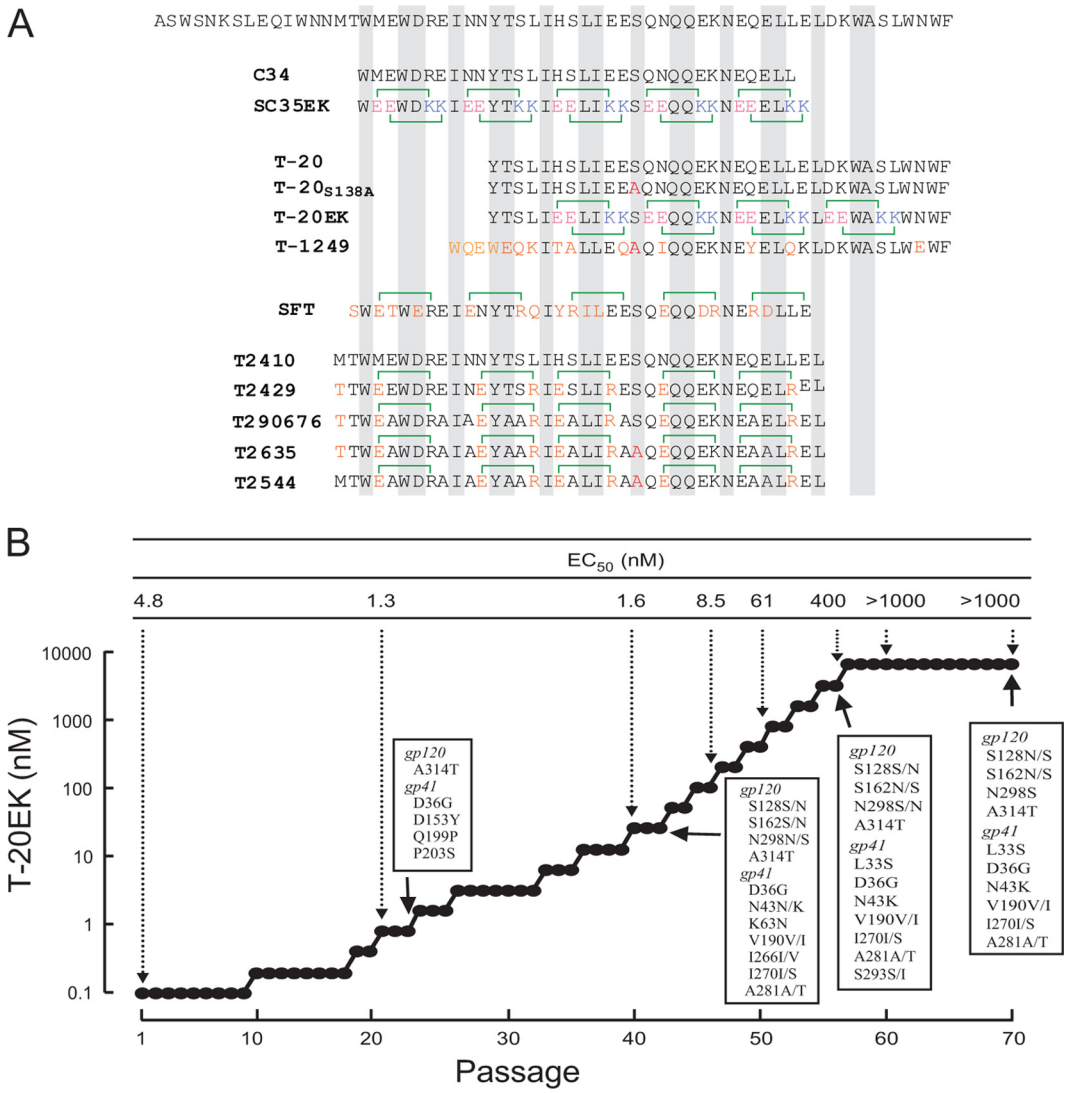

FIG 1 (A) Amino acid sequences of fusion-inhibitory peptides used in this study. The HIV-1 C-HR amino acid sequence is shown in the first row. Electrostatic interactions are indicated in pink and light blue for acidic (glutamic acid [E]) and basic (lysine [K]) residues, respectively. Modified amino acids are indicated in orange. A resistance-associated substitution, S138A, is indicated in red. Amino acids for the interactive site are shaded gray. (B) The dose escalation method for selection of T-20EK resistance through passage in MT-2 cells. Induction of resistant HIV-1 was performed over a total of 70 passages from 0.1 nM T-20EK. At the indicated passage, proviral DNAs were sequenced, and the 50% effective concentrations (EC_{50}s) of the HIV-1 variants were determined in a MAGI assay. All substitutions shown in boxes were observed in combination.

tivation of a β-galactosidase indicator) assay (10, 25, 26). Our data revealed that L33S and N43K are major primary substitutions for T-20EK resistance (Table 1). Substitutions in the CT domain weakly enhanced the resistance induced by L33S/N43K. We previously showed that the S138A substitution in T-20 ($T\text{-}20_{S138A}$) leads to substantial inhibition of the T-20-resistant variant $HIV\text{-}1_{L33S/N43K}$ (10, 14, 23). Interestingly, $T\text{-}20EK_{S138A}$, a variant of T-20EK that is expected to exert strong activity to resistant variants, did not inhibit efficiently $HIV\text{-}1_{L33S/N43K}$ (Table 1). We found cross-resistance between T-20EK and other T-20-based fusion inhibitors, except for T1249, which has an amino acid sequence that overlaps with T-20 (Fig. 1A). In contrast, C34 and its derivatives maintained their activity to T-20EK-resistant variants. These results indicated that T-20EK may show cross-resistance only with T-20-derived peptides and that the mechanisms of resistance to T-20 and C34 derivatives are different.

During the selection, we observed one substitution in the gp41 transmembrane domain, V190I, and two in the cytoplasmic domain (intravirion), I270S and A281T (Fig. 1B). These three substitutions are also observed in T-20-naive isolates (Los Alamos National Library, HIV Sequence Database; http://www.hiv.lanl.gov). Although these substitutions contributed little to the resistance (Table 1), I270S/A281T substitutions in gp41 cytoplasmic tail restored significantly reduced replication kinetics by substitutions in the ectodomain and transmembrane domain (see Fig. S2 in the supplemental material). Other substitutions, K63N, D153Y, Q199P, P203S, I266V, and S293I, were transiently observed but later disappeared during the selection (see Fig. S1 in the supplemental material). K63N is located adjacent to Q64, which was previously shown to be a resistance-associated substitution (32). The synonymous change in V72 (GTG to GTA) may influence the RRE structural stability, as we have previously described (28).

TABLE 1 Susceptibilities of HIV-1 recombinant clones with substitutions in gp41 to peptide fusion inhibitors[a]

Peptide	EC_{50} (nM) of HIV-1 clone				
	HIV-1_{WT}[b]	HIV-1_{L33S}	HIV-1_{N43K}	HIV-$1_{L33S/N43K}$	HIV-$1_{L33S/N43K/T\text{-}CT}$[c]
Dideoxycytosine	356 ± 89	347 ± 129 (1.0)	489 ± 37 (1.4)	431 ± 75 (1.2)	474 ± 273 (1.3)
T-20-based peptides					
T-20	2.5 ± 0.2	66 ± 9 (**26**)	31 ± 4 (**12**)	>1,000 (>**400**)	>1,000 (>**400**)
$T\text{-}20_{S138A}$	0.55 ± 0.14	3.2 ± 0.7 (5.8)	0.45 ± 0.07 (0.8)	30 ± 10 (**55**)	73.3 ± 23 (**133**)
T-20EK	1.2 ± 0.3	12 ± 2 (**10**)	12 ± 1 (**10**)	>1,000 (>**833**)	>1,000 (>**833**)
$T\text{-}20EK_{S138A}$	0.43 ± 0.06	2.2 ± 0.4 (5.1)	2.3 ± 0.7 (5.4)	112 ± 20 (**260**)	301 ± 110 (**700**)
T1249	0.29 ± 0.07	0.31 ± 0.10 (1.1)	0.35 ± 0.10 (1.2)	0.60 ± 0.16 (2.2)	ND
C34-based peptides					
C34	1.1 ± 0.2	2.9 ± 0.4 (2.6)	2.6 ± 0.7 (2.4)	4.1 ± 1.6 (3.7)	9 ± 5.2 (8)
SC35EK	1.7 ± 0.4	1.8 ± 0.3 (1.1)	2.4 ± 0.5 (1.4)	2.5 ± 0.6 (1.5)	3.4 ± 1.9 (2)
Sifvirtide	2.1 ± 0.4	2.5 ± 0.2 (1.2)	1.5 ± 0.3 (0.7)	2.2 ± 0.5 (1.0)	ND
T2410	1.1 ± 0.3	0.64 ± 0.23 (0.6)	1.8 ± 0.5 (1.6)	1.4 ± 0.5 (1.3)	ND
T2429	1.9 ± 0.3	1.9 ± 0.5 (1.0)	2.5 ± 0.8 (1.3)	1.8 ± 0.1 (0.9)	ND
T290676	0.46 ± 0.14	0.54 ± 0.12 (1.2)	0.46 ± 0.10 (1.0)	1.1 ± 0.5 (2.4)	ND
T2635	0.43 ± 0.02	0.55 ± 0.22 (1.3)	1.4 ± 0.6 (3.3)	0.57 ± 0.22 (1.3)	1.7 ± 0.4 (4)
T2544	0.46 ± 0.08	1.3 ± 0.3 (2.8)	2.4 ± 0.4 (5.2)	1.1 ± 0.4 (2.4)	ND

[a] Drug susceptibilities were determined in a MAGI assay and are reported here as means ± standard deviations from at least three independent assays. Values in parentheses are the fold change in resistance compared with HIV-1_{WT}. Peptide fusion inhibitors that caused more than 10-fold resistance are indicated in bold. ND, not determined.
[b] $NL4.3_{D36G}$ was defined as wild-type HIV-1 (HIV-1_{WT}).
[c] T-CT (transmembrane and cytoplasmic tail domains) indicates the substitutions V190I, I270S, and A281T, which were only observed in a mixture.

With the exception of D153Y, Q199P, and P203S in gp41, all substitutions were observed in the vast majority of T-20-naive patients, indicating that they are natural polymorphisms. P203S was also selected as a low-level SC34EK resistance-associated substitution (26). This is consistent with our data showing that combinations of CT substitutions only enhance resistance by 2- to 3-fold (Table 1). Moreover, most substitutions coexisted with variants containing the wild-type sequence, strongly indicating that they, as well as those in gp120, exerted only a modest effect on resistance.

We previously demonstrated that T-20EK inhibited T-20-resistant variants harboring G36D, V38A, or N43D/K substitutions (28) and that it maintains its strong antiviral effect against HIV-2 (23). T-20EK-resistant variants showed very limited cross-resistance to other novel fusion inhibitors, with the exception of T-20-based peptides, indicating that the combination of T-20EK with other new fusion inhibitors may be suitable for therapy. The enhanced hydrophilicity of T-20EK by the engineered hydrophilic amino acids (Glu and Lys) is a favorable property for solubility, which is expected to reduce some of the adverse effects of T-20, such as skin reactions at the injection site (33). Interestingly, our experiments did not result in the selection of any secondary substitutions in the C-HR, such as N126K and S138A, that are frequently observed in variants that are resistant to novel fusion inhibitors (26, 34, 35). In contrast, one of the primary substitutions was L33S, a substitution at a site outside the N-HR. Notably, the replication kinetics of L33S are comparable to those of wild-type HIV-1 (unpublished data). Therefore, L33S seems to be a T-20-specific substitution that does not require the presence of secondary substitutions. Importantly, use of T-20EK does not lead to the appearance of substitutions that confer cross-resistance to other novel fusion inhibitors. Thus, our study establishes that T-20EK can become an efficient new fusion inhibitor.

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