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

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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 abort fusion 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 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. 1A) 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. 1B).

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. 1B), 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-
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 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.

**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.

**Application of Resistant Mutations to Enfuvirtide**

**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 multienzyme activation of galactosidase indicator (MAGI) assay as described previously (13). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^5 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, EC50).

**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 TransIT® (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, Tm) 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-
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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.

| Peptide         | EC_{50} (nM) | HIV-1_{WT}^a | HIV-1_{V36A} | HIV-1_{N43D} | HIV-1_{N43D/S138A} |
|-----------------|------------|-------------|-------------|-------------|-------------------|
| 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_{S138W}    | 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}    | 416 ± 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_{S138V}    | 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).

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. 1B). 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_{V36A}
or N36 N43D, sufficient α-helicity at 25 °C was observed only in C34S138A, C34S138L, and C34S138T or C34S138A, C34S138L, and C34S138W, 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 (Tm) 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 (Tm) indicating the 50% disruption of 6-helix bundle was comparatively evaluated. Complexes of N36 and C34 containing the S138A or S138L substitutions (N36/C34S138A or N36/C34S138L) 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 N36N43D/C34 complex. These results reveal a striking correlation between the thermal stability and the anti-HIV-1 activity of the complexes (R² = 0.75, Fig. 3). The low Tm value of the complex formed from N36N43D 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 C34S138A and C34S138L peptides showed potent anti-HIV-1 activities, similar to T-20S138A and T-20S138L (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-20S138X 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 C34N126K 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. 1B), 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 derivative peptide that has S138A substitutions. The C34S138A and C34S138L peptides showed potent anti-HIV-1 activities, similar to T-20S138A and T-20S138L (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-20S138X to N-HR.

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TABLE 2

| HIV-1WT | HIV-1 V4/I37K/N126K/L204I |
|---------|---------------------------|
| C34 | 1.6 ± 0.35 | 114 ± 29 (71) |
| C34S126K | 0.95 ± 0.22 (0.6) | 1.1 ± 0.5 (0.7) |

* 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).
| 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.

** TABLE 2 **

Antiviral activity of C34N126K 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 EC₅₀ value) calculated by comparison to a reference virus. The increase of >10-fold is indicated in bold.

| EC₅₀ nM | |
|---------|---|
| HIV-1WT | 1.6 ± 0.35 |
| HIV-1 ΔV4/I37K/N126K/L204I | 114 ± 29 (71) |

| C34S126K | 0.95 ± 0.22 (0.6) |
|----------|-------------------|
| 1.1 ± 0.5 (0.7) |
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. Syncitia 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.

**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 reside 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.

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-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-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_{\alpha}, 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.

REFERENCES

1. Chan, D. C., and Kim, P. S. (1998) Cell 93, 681–684
2. Weiss, C. D. (2003) AIDS Rev. 5, 214–221
3. Jiang, S., Lin, K., Strick, N., and Neurath, A. R. (1993) Nature 365, 113
4. Chan, D. C., Chutkowski, C. T., and Kim, P. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15613–15617
5. Dwyer, J. J., Wilson, K. L., Davison, D. K., Freal, S. A., Seedorf, J. E., Wring, S. A., Tvermoes, N. A., Matthews, T. J., Greenberg, M. L., and Delmedico, M. K. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 12772–12777
6. Otaka, A., Nakamura, M., Nameki, D., Kodama, U., Uchiyama, S., Naka­mura, S., Nakano, H., Tamamura, H., Kobayashi, Y., Matsuoka, M., and Fuji­ji, N. (2002) Angew. Chem. Int. Ed. Engl. 41, 2937–2940
7. Lalezari, J. P., Henry, K., O’Heaen, M., Montaner, J. S., Pillerio, P. I., Trot­tier, B., Walmsley, S., Cohen, C., Kuritzkes, D. R., Eron, J. J., Liu, Chu­ng, J., DeMa­si, R., Donatacci, L., Drobnis, C., De­lehan­ty, J., and Sol­go, M. (2003) N. Engl. J. Med. 348, 2175–2185
8. Lazzarin, A., Clet­ot, B., Cooper, D., Reyes, J., Arasteh, K., Nelson, M., Katlama, C., Stellbrink, H. J., Delfraissy, J. F., Lange, J., Huson, L., De­Ma­si, W., Wat, C., De­lehan­ty, J., Dro­busc­es, C., and Sol­go, M. (2003) N. Engl. J. Med. 348, 2186–2195
9. Bald­win, C. E., and Ber­k­hout, B. (2006) Retrovi­rology 3, 84
10. Ca­brera, C., Mar­fil, S., Gar­cia, E., Marti­nez­Pica­do, J., Bon­joch, J., Bof­fill, M., More­no, S., Ribere, E., Domingo, P., Clet­ot, B., and Ru­iz, L. (2006) AIDS 20, 2075–2080
11. Lab­rosse, B., Morand­Joubert, L., Gou­bard, A., Rochas, S., Lab­ernardiere, J. L., Pacanow­ski, J., Meye­nard, J. L., Hance, A. J., Clavel, F., and Mam­mno, M. K. (2007) J. Virol. 80, 8807–8819
12. Mink, M., Mosier, S. M., Janum­palli, S., Davi­son, D., Jin, L., Mel­by, T., Si­sta, P., Erick­son, J., Lambert, D., Stan­field­Oak­ley, S. A., Sal­go, M., Cam­mack, N., Ma­thews, T., and Green­berg, M. L. (2005) J. Virol. 79, 12447–12454
13. Nameki, D., Kodama, E., Ikeuchi, M., Mabu­chi, N., Otaka, A., Tamarama, H., Oh­no, M., Fuji­ji, N., and Matsuoka, M. (2005) J. Virol. 79, 764–770
14. Per­ez-Alba­rez, L., Car­ma­na, R., Oco­malo, A., Asorey, M., Miralles, C., Perez de Cas­tro, S., Pinilla, M., Con­treras, G., Taboada, J. A., and Na­jera, R. (2006) J. Med. Virol. 78, 141–147
15. Xu, L., Poz­niak, A., Wild­fire, A., Stan­field­Oak­ley, S. A., Mosier, S. M., Rat­cli­ffe, D., Work­man, J., Jo­all, A., My­ers, R., Smit, E., Can­e, P. A., Green­berg, M. L., and Pilla­y, D. (2005) Antimicrob. Agents Chemother. 49,
