In Vitro Selection and Characterization of HIV-1 Variants with Increased Resistance to Sifuvirtide, a Novel HIV-1 Fusion Inhibitor*

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Sifuvirtide, a novel fusion inhibitor against human immunodeficiency virus type 1 (HIV-1), which is more potent than enfuvirtide (T20) in cell culture, is currently under clinical investigation for the treatment of HIV-1 infection. We now report that in vitro selection of HIV-1 variants resistant to sifuvirtide in the presence of increasing concentrations of sifuvirtide has led to several specific mutations in the gp41 region that had not been previously reported. Many of these substitutions were confined to the N-terminal heptad repeat region at positions 37, 38, 41, and 43, either singly or in combination. A downstream substitution at position 126 (N126K) in the C-terminal heptad repeat region was also found. Site-directed mutagenesis studies have further identified the critical amino acid substitutions and combinations thereof in conferring the resistant genotypes. Furthermore, the mutant viruses demonstrated variable degrees of cross-resistance to enfuvirtide, some of which are preferentially more resistant to sifuvirtide. Impaired infectivity was also found for many of the mutant viruses. Biophysical and structural analyses of the key substitutions have revealed several potential novel mechanisms against sifuvirtide. Our results may help to predict potential resistant patterns in vivo and facilitate the further clinical development and therapeutic utility of sifuvirtide.

The envelope glycoprotein (Env) of HIV-1 is critical in mediating viral entry into the target cells, and it represents a major target for the development of novel antiretroviral therapeutics. The entry process starts with the binding of gp120 to a cellular receptor, CD4, and subsequently with a chemokine receptor, CCR5 or CXCR4, on the surface of the target cells (1). These interactions trigger a cascade of conformational changes that lead to the formation of a prehairpin intermediate of gp41 in which the hydrophobic N-terminal heptad repeat (NHR) is exposed and allows the fusion peptides to insert into the target cell membrane (1, 2). This transient gp41 intermediate then refolds into a stabilized trimer of hairpins, also called the six-helix bundle (6-HB) structure, which brings the viral envelope and the target cell membrane into close proximity, thus facilitating the completion of the fusion process (1–4). Structure and function studies indicate that the 6-HB core consists of a parallel trimeric coiled-coil of NHR with the C-terminal heptad repeat (CHR) wrapped on the outside in an antiparallel fashion (5–7). Similar features have been found in the fusion-mediating subunits of other viruses with class I membrane fusion proteins (8–17).

Inhibitors capable of disrupting the entry process hold great promise for improved efficacy of clinical antiviral therapeutics. Several entry inhibitors have been developed, and two of these have been approved for clinical use, such as a peptide-based inhibitor enfuvirtide (T20) and a small molecule inhibitor Maraviroc against HIV-1 co-receptor CCR5 (18–20). In addition, several other entry inhibitors are being developed. These include the co-receptor antagonist small molecule inhibitor vicriviroc, the anti-CCR5 monoclonal antibody (21, 22), the anti-human CD4 monoclonal antibody ibalizumab (TNX-355) (23), and new generations of peptide-based and small molecule inhibitors targeting the fusion process (24–28). We have previously characterized a new generation peptide-based fusion inhibitor known as sifuvirtide, which has an improved stability, pharmacokinetics, and antiviral potency, as compared with enfuvirtide (29).

In this report, we describe the in vitro selection and characterization of HIV-1 variants with relative resistance to sifuvirtide. We derived resistant variants of HIV-1 NL4-3 by serial passage in the presence of increasing concentrations of sifuvirtide. By sequencing analysis of resistant variants, we have identified several novel specific mutations in the NHR and CHR regions in gp41 associated with observed resistant phenotypes. Site-directed mutagenesis studies have further identified the critical amino acid substitutions in conferring the resistant genotypes to sifuvirtide and cross-resistance to enfuvirtide. Finally, biophysical and structural analyses of these substitutions have revealed several potential mechanisms against sifuvirtide. These results may help to predict

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‡‡‡ The abbreviations used are: NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; 6-HB, six-helix bundle.
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potential patterns of resistance in vivo and may facilitate the further clinical development and therapeutic utility of sifuvirtide.

EXPERIMENTAL PROCEDURES

Selection of Sifuvirtide-resistant HIV-1 Variants—MT4 cells were seeded at 3 × 10^5/ml in RPMI 1640 medium containing 10% fetal bovine serum on a 96-well plate. Serial dilutions of wild-type virus, molecular clone NL4-3, were added and followed by incubation at 37 °C with 5% CO₂ for 5 days. The concentration of sifuvirtide required to inhibit 50% viral infection was calculated based on the cytopathic effect. For selection of sifuvirtide-resistant virus, we initially used 7.8 ng/ml sifuvirtide for wild-type virus, which can inhibit virus replication by about 90%. Cells were incubated at 37 °C with 5% CO₂ until extensive cytopathic effect was observed, and supernatants were used for the next passage in MT4 cells with 1.5–2-fold increasing concentrations of sifuvirtide. During the course of selection, wild-type virus was used without sifuvirtide as a parallel control. After 15 passages, the virus isolates were able to grow at a sifuvirtide concentration up to 5 μg/ml.

Proviral DNA Isolation, PCR Amplification, and Sequencing—Cells were pelleted by centrifugation, and DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen). The full-length gp41 gene was amplified from cellular DNA with primers (forward primer, 5′-TGGAGGAGGCGATATGAGGG-3′; reverse primer, 5′-GATAGTGGAGCCTGGTAG-3′) using the Platinum® TaqDNA polymerase high fidelity PCR system (Invitrogen). The PCR products were analyzed on 1% agarose gel and then purified by the QIAquick gel extraction kit (Qiagen). PCR products were sequenced and analyzed by BioEdit software (available on the World Wide Web). Construction of pNL4-3 Mutant Molecular Clones—Plasmid pNL4-3 containing the full-length genome of HIV-1 (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health) was digested with restriction endonucleases NheI and BamHI to release the fragment containing the full-length gp41 and cloned into the vector pVAX1/lacZ (Invitrogen). Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) verified by direct sequencing, and the fragments containing mutations were cloned back to original NL4-3.

Transfection and p24 Determination—The virus stock for wild type and mutant viruses containing various mutations were produced by transfecting 293T cells using FuGENE®6 transfection reagent (Roche Applied Science). The supernatants containing infectious wild-type and mutant viruses were harvested after a 48-h incubation. The concentration of p24 of each virus was measured by an HIV-1 antigen kit (Vironostika, Netherlands) and stored at −80 °C until use.

IC₅₀ and Infectivity Determination—We used TZM-bl cells (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health) to study viral infectivity. These cells are genetically engineered to stably express high levels of CD4 and HIV-1 co-receptors CCR5 and CXCR4 and also to contain the luciferase and β-galactosidase genes under the control of the HIV-1 long terminal repeat promoter. Approximately 3 × 10⁴ cells/well were plated on a 96-well plate in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine and penicillin/streptomycin and then incubated at 37 °C with 5% CO₂. Serial dilution of sifuvirtide or enfuvirtide was added to the cells. One nanogram of p24 equivalent viral stock was added, and each concentration of sifuvirtide or enfuvirtide was assayed in triplicate. Forty-eight hours postinfection, 50 μl of supernatants were discarded, and 50 μl of Bright Glo reagent (Promega) was added to the each well. After a 5-min incubation at room temperature to allow cell lysis, 100 μl of cell lysate was transferred to 96-well black solid plates for measurements of luminescence using a Berthold Centro LB 960 luminometer (Berthold Technologies). Nonlinear regression curves were generated, and 50% inhibitory concentration (IC₅₀) was calculated using Prism software version 4.0 (GraphPad Software).

Peptide Synthesis and Characterization—Sifuvirtide peptide was kindly provided by Dr. Genfa Zhou (FusoGen Pharmaceuticals, Inc., Tianjin, China). C34, N36, and mutant N peptides were synthesized by a standard solid-phase Fmoc (N-(9-fluorenylmethoxycarbonyl) method using a ThuraMed TETRAS synthesizer (Louisville, KY). All peptides were acetylated at the N termini and amidated at the C termini. The peptides were purified to homogeneity (>95% purity) by HPLC (Agilent 1200) and further analyzed by laser desorption mass spectrometry (Waters, Milford, MA). The concentration of peptides was determined by UV absorbance and a theoretically calculated molar extinction coefficient ε (280 nm) of 5500 mol/liter−1 cm⁻¹ and 1490 mol/liter−1 cm⁻¹ based on the number of tryptophan residues and tyrosine residues (all of the tested peptides contain Trp and/or Tyr).

Native PAGE—Native PAGE was carried out to determine the 6-HB formation between the C and N peptides as described previously (30).

Analysis of 6-HB Formation by ELISA—The ability of various mutant N peptides to form 6-HB with C34 was measured by an ELISA (31) with modification.

CD Spectroscopy—The secondary structure of the peptides and their mixtures was analyzed by CD spectroscopy as described previously (32, 33).

Structural Modeling and Analysis—The structural modeling of interaction between mutant N peptides (including Q41K, Q41H, Q41R, N43K, I37T, and V38A) and C peptide (C34 or mutant N126K) was based on the trimeric structure recently obtained from HIV-1 HXB2 (Protein Data Bank code 2X7R) (34). The updated structure contains both fusion peptide and membrane-proximal external regions of gp41 and represents the most complete example of interaction between the N peptide and C peptide (34). The 6-HB for each mutant peptide was constructed through symmetry operation in alignment mode using the automated protein modeling program on the SWISSMODEL protein-modeling server (35).

RESULTS

In Vitro Selection of Viruses Resistant to Sifuvirtide—HIV-1 isolates resistant to sifuvirtide were generated and selected by serial passage of molecular clone HIV-1 NL4-3 through MT-4
cells with increasing concentrations of sifuvirtide. A total of 12 independent cultures were conducted with a starting concentration of sifuvirtide at 7.8 ng/ml, which is sufficient to inhibit about 90% of virus replication. About 1 ng of p24 equivalent of HIV-1 NL4-3 was added to the MT-4 cells 1 h before the addition of sifuvirtide. Half of the culture medium was replaced every other day, and sifuvirtide with appropriate concentration was maintained in the culture medium throughout the passage process. Cells were routinely monitored for cytopathic effect. Supranatant in which the cytopathic effect was present was collected and continued to culture with a 2-fold higher concentration of sifuvirtide. After 9–15 passages, all 12 HIV-1 isolates continued to replicate in the presence of sifuvirtide up to $5^{1/2} /H9262$ g/ml, although variability existed among different isolates on the detectable levels of cytopathic effect. The marked increase in sifuvirtide concentration suggests the presence of highly resistant variants in the viral culture.

Phenotypic Resistance to Sifuvirtide Is Associated with Multiple Genotypic Changes in the NHR of gp41—To study the genetic changes associated with resistance to sifuvirtide, proviral DNAs from infected cells of all 12 independent cultures at the last passages were subjected to PCR to amplify the entire gp41 region. As shown in the upper panel of Fig. 1, several amino acid substitutions were identified in the NHR region, some of which have been identified previously against C34, SC34, and SC34-EK (36, 37), whereas some or a combination thereof have not been reported previously. No substitutions were found in the pocket-binding domain despite the ubiquitous presence of sifuvirtide (Fig. 1, top). These substitutions are located at positions 37, 38, 41, and 43, either singly or in combination. Notably, all substitutions at position 37 involve the change of isoleucine to threonine (I37T, 6 of 12) (1a, 1b, 1c, 2c, 3b, and 4b), whereas the substitution at position 43 produces the change from asparagine to lysine (N43K, 5 of 12) (1b, 1c, 2c, 3a, and 4a). Substitutions at position 38 are more variable, from valine to either methionine (V38M, 2 of 12) or alanine (V38A, 1 of 12). Substitutions at position 41 are unique in that many of the mutants bear positively charged residues, such as arginine (Q41R, 2 of 12), lysine (Q41K, 2 of 12), or histidine (Q41H, 1 of 12), which have only been reported a few times among over 2229 standard HIV-1 sequences in the database (available on the World Wide Web). Furthermore, analysis of combinational substitutions revealed that double mutations at positions 37 and 43 (I37T/N43K) are the most frequent, having been identified in three (1b, 1c, and 2c) of the 12 independent cultures (Fig. 1). The paired substitutions have so far not been reported in naturally infected or treated patients or in any in vitro culture resistant to other peptide-based fusion inhibitors against HIV-1. Triple mutations were also identified in two cultures (3b and 4a), both of which share the positively charged residues at position 41 (Fig. 1). Sporadic mutations at positions 71 and 113 involving alanine to threonine (A71T) and asparagine to aspartic acid
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**TABLE 1**

| Culture number | Amino acid substitution(s) | Codon change |
|----------------|--------------------------|--------------|
| 1a             | I37T/N126K               | ATA→ACA/AAT→AAA |
| 1b             | I37T/N43K                | ATA→ACA/AAT→AAA |
| 1c             | I37T/N43K/A71T/N113D/N126K | ATA→ACA/AAT→AAA/GCT→ACT/AAT→GAT/AAT→AAG |
| 2a             | V38M                     | GTG→ATG       |
| 2b             | Q41H                     | CAG→CAC       |
| 2c             | I37T/N43K/N126K          | ATA→ACA/AAT→AAA/AAT→AAG |
| 3a             | N43K                     | AAT→AAA       |
| 3b             | I37T/V38A/Q41R/N126K     | ATA→ACA/GTG→GGC/CAG→CGG/AAT→AAA |
| 3c             | Q41R/N126K               | CAG→CGG/AAT→AAA |
| 4a             | V38M/Q41K/N43K/N126K     | GTG→ATG/CAG→AAG/AAT→AAA/AAT→AAG |
| 4b             | I37T                     | ATA→ACA       |
| 4c             | Q41K                     | CAG→AAG       |

**TABLE 2**

| Mutant viruses | Sifuvirtide IC50 (S.D.) | Resistance | Enfuvirtide IC50 (S.D.) | Resistance |
|----------------|-------------------------|------------|-------------------------|------------|
|                | mM                      | fold       | mM                      | fold       |
| NL4–3          | 2.87 (0.36)             | 1.00       | 229.44 (30.11)          | 1.00       |
| I37T           | 15.31 (0.38)            | 5.33       | 1677.75 (379.55)        | 7.31       |
| V38A           | 18.11 (1.28)            | 6.33       | 3520.67 (481.12)        | 15.34      |
| V38M           | 9.22 (1.53)             | 3.21       | 1549.89 (111.69)        | 6.76       |
| Q41K           | 114.92 (5.67)           | 40.04      | 3889.44 (1711.46)       | 16.95      |
| Q41H           | 5.28 (3.32)             | 1.84       | 800.22 (444.04)         | 3.49       |
| Q41R           | 80.96 (9.71)            | 28.21      | 4093.93 (715.51)        | 17.84      |
| N43K           | 27.61 (1.67)            | 9.62       | 1462.02 (262.25)        | 6.37       |
| A71T           | 5.71 (1.02)             | 1.99       | 301.57 (24.27)          | 1.31       |
| N113D          | 1.95 (1.43)             | 0.68       | 298.2 (112.13)          | 1.30       |
| N126K          | 10.83 (1.47)            | 3.77       | 471.24 (57.3)           | 2.05       |
| I37T/V38A      | 126.59 (30.98)          | 44.10      | 8301.35 (2246.29)       | 36.18      |
| I37T/N43K      | 277.85 (25.96)          | 96.80      | 2182.47 (696.63)        | 9.51       |
| I37T/N43K/A71T | 248.61 (2.58)           | 86.62      | 2973.93 (417.3)         | 12.96      |
| I37T/N43K/N126K| 439.18 (30.05)          | 153.01     | 1916.4 (937.08)         | 8.35       |
| I37T/N43K/N113D| 256.51 (29.3)           | 89.37      | 4629.89 (372.13)        | 20.18      |
| I37T/N43K/A71T/N126K| 419.97 (125.79)       | 146.32     | 2014.38 (339.55)        | 8.78       |
| I37T/N43K/A71T/N113D/N126K| 590.06 (114.53)       | 205.58     | 4807.87 (189.89)        | 20.95      |
| V38A/Q41R      |                         |            |                         |            |
| V38M/Q41K      |                         |            |                         |            |
| I37T/V38A/Q41R |                         |            |                         |            |

(N113D), respectively, were identified only for clone 1c (data now shown). Last, a downstream substitution from asparagine to lysine at position 126 within the CHR region was also observed in five of the 12 cultures (1a, 1c, 2c, 3b, 3c, and 4a) (data not shown). Codon changes at the nucleotide level leading to the non-synonymous substitutions shown in Fig. 1 are listed in boldface italic type in Table 1. No preferences were noted toward either transition or transversion.

**Key Mutations Determined by Site-directed Mutagenesis**—To study which mutation(s) play the dominant role in conferring observed resistance to sifuvirtide, we have introduced each individual mutation, as well as double and multiple mutations, into the NHR region of gp41 and further cloned into the backbone of wild-type molecular clone NL4-3. All 12 mutant clones identified in the culture (1a–4c), as well as some additional single mutants, were constructed to study the relative contribution of individual mutations to the overall resistance. A total of 22 mutant clones were constructed, and their sequences were confirmed by sequencing before generating the viral stock for infection analysis (Table 2). Fig. 2 shows the drug sensitivity data to sifuvirtide of nine representative mutant clones. It is evident that among all of the single mutants, there is a clear trend of increase in the level of resistance from Q41H, V38M, I37T, V38A, N43K, Q41R, and Q41K relative to the wild-type NL4–3 (Table 2 and Fig. 2). Substitutions at position 41 result in dramatic differences in resistance, with Q41K the strongest, Q41R intermediate, and Q41H the least resistant (Fig. 2 and Table 2). For the double mutants, both I37T/N43K and I37T/V38A demonstrated significant increases in resistance (Fig. 2 and Table 2, middle). However, substitution N43K clearly contributes more than V38A in the context of I37T background. Last, triple and multiple substitutions have clear added benefit to the viruses upon challenge by sifuvirtide.

Most notable is the substitution N126K, which elevates the resistance level dramatically in the context of I37T/N43K, whereas the impact of sporadic substitutions, such as A71T and N113D, is rather minimal (Table 2, bottom). It should be noted that mutants with substitutions identical to culture 3b and 4a and other clones bearing substitutions in addition to that at position 41 (Q41K or Q41R) resulted in defective viruses, which failed to infect the target cells despite multiple attempts (Table 2, bottom).

Taken together, our results suggest that resistance to sifuvirtide is most likely a result of multiple different substitutions, either alone or in combination, leading to different re-
Cross-resistance mechanisms. Single substitution at position 41 (Q41K or Q41R) and double substitutions I37T/N43K or I37T/V38A in combination with N126K are probably the key mutations responsible for observed increases in resistant phenotype.

Cross-resistance of Mutant Viruses to Enfuvirtide—A cross-resistance study was further conducted against the clinically approved peptide-based inhibitor enfuvirtide. All mutant clones, together with wild-type molecular clone NL4-3, were subjected to serial concentrations of either enfuvirtide or sifuvirtide in the cell supernatant. Their sensitivity to inhibitions by either enfuvirtide or sifuvirtide and -fold increases relative to wild-type NL4-3 are summarized in Table 2. In terms of absolute molar concentration, sifuvirtide is close to 80-fold more potent than enfuvirtide in inhibiting wild-type NL4-3, consistent with our previous observation (29). For cross-resistance analysis, we focused more on the -fold increases and investigated whether the same substitutions resulted in different resistance variation against sifuvirtide and enfuvirtide. For all mutants with single substitutions, there is a good correlation between -fold increases for sifuvirtide and enfuvirtide. The most evident are the Q41K and Q41R substitutions, which result in double-digit increases toward both sifuvirtide (40.04 and 28.21) and enfuvirtide (16.95 and 17.84) (Table 2, top). Q41H substitution, despite being at the same location, had only minimal impact on cross-resistance (1.84 versus 3.49) (Table 2, top). Substitutions at position 37, 38, or 43 share a similar level of cross-resistance at the single-digit level, although the V38A mutant is probably more resistant to enfuvirtide than to sifuvirtide (15.34 versus 6.33), whereas the N43K mutant seems to be more resistant to sifuvirtide (9.62 versus 6.37) (Table 2, top). Downstream substitutions, such as A71T and N113D, have negligible impact on overall resistance, whereas minor increases in cross-resistance were observed for the mutant with N126K substitution (3.77 versus 2.05) (Table 2, top). For mutants with double substitutions, I37T/V38A resulted in similar level of increase in resistance to sifuvirtide and cross-resistance to enfuvirtide (44.10 versus 36.18) (Table 2, middle), which is quite comparable with resistance conferred by single substitutions Q41K and Q41R (see above). Substitution I37T/N43K, however, preferentially shows more resistance to sifuvirtide than cross-resistance to enfuvirtide, and the difference reached over more than 10-fold (96.80 versus 9.51) (Table 2, middle). For mutants with triple substitutions, it is clear that the additional downstream substitution of N126K in the context of I37T/N43K has led to three-digit increases in resistance against sifuvirtide but only minimal cross-resistance to enfuvirtide (153.01 versus 8.35) (Table 2, bottom). Although additional single A71T or N113D substitution had no added resistance to sifuvirtide, there was a trend toward more cross-resistance to enfuvirtide (Table 2, bottom). Finally, increases in resistance for mutants with four or more substitutions are quite comparable with those of triple mutants, although both a slightly higher resistance and

![Figure 2](image-url)
cross-resistance were noticed for the mutant with I37T/N43K/A71T/N113D/N126K substitutions in combination (Table 2, bottom).

**Relative Infectivity of Mutant Clones**—To measure the infectivity of mutant clones, we have performed an infection assay in the absence of sifuvirtide or other inhibitors using the TZM-bl reporter cell line. One nanogram of p24 equivalent of each mutant virus, together with wild-type NL4-3, was used to infect the target cells, and luciferase activity was measured 2 days after infection. Infectivity of NL4-3 was normalized to 100%, and the relative infectivity of other mutants was calculated accordingly. For mutants with single substitutions, I37T resulted in a 50% increase in infectivity, whereas Q41H remained similar to that of the wild-type NL4-3 (Fig. 3). In contrast, other mutant viruses revealed variable and diminished infectivity. Some substitutions, such as Q41K, Q41R, and V38M, severely reduced infectivity more than 50%, suggesting the critical role of these residues for viral infection. For mutant viruses with double substitutions, I37T/V38A resulted in an ~50% increase in infectivity, whereas Q41H remained similar to that of the wild-type NL4-3 (Fig. 3). In contrast, other mutant viruses revealed variable and diminished infectivity. Some substitutions, such as Q41K, Q41R, and V38M, severely reduced infectivity more than 50%, suggesting the critical role of these residues for viral infection. For mutant viruses with double substitutions, I37T/V38A resulted in an ~50% increase in infectivity, whereas Q41H remained similar to that of the wild-type NL4-3. Mutant viruses with double substitutions, I37T/N43K revealed infectivity closer to wild-type NL4-3 (Fig. 3). For mutant viruses with triple or more substitutions, all demonstrated decreased infectivity to varying degrees. Mutant virus with I37T/N43K/N126K substitution, however, was the most fit among them all (Fig. 3). Again, it should be noted that among the 22 mutant viruses generated, five with double or more substitutions were defective in entering the cells. Close analysis of these clones revealed a common feature where positively charged substitutions at position 41 (Q41K or Q41R) were found (Table 2, bottom). Because single substitution of either Q41K or Q41R already resulted in profound reduction in viral infectivity, it is possible that any additional substitutions become intolerable to the viruses. This finding indicates that the residue at position 41 is functionally critical to the virus and explains its high degree of conservation among viruses identified so far.

**Characterization of 6-HB Formation by the Mutant Peptides**—We previously developed a native PAGE-based technique to visualize 6-HB formation by N36/C34 and used it to characterize the antiviral activity of HIV-1 fusion inhibitors (30, 38, 39). In this study, we applied this technique to study the interaction between the peptides bearing the key resistant substitutions and C34 and their impact on the formation of 6-HB. Six 36-mer N peptides bearing the key substitutions at position 37, 38, 41, or 43 were synthesized, four of which have a single substitution (I37T, V38A, Q41K, and N43K) and the remainder of which have two substitutions (I37T/V38A and I37T/N43K). As shown in Fig. 4A, none of the N peptides alone, including the wild-type N36, shows visible bands on the gel because they carry net positive charges and may migrate up and off the gel. Peptide C34 alone, however, displayed a band in the lower part of the gel. Furthermore, like wild-type N36, all mutant peptides carrying the key substitutions were able to form 6-HB with C34, reflected by the up-shifted bands for each mixing complex compared with C34 alone (Fig. 4A). Some variations in density were found for the shifted band among different complexes, suggesting different efficiency and capacity in 6-HB formation among the different mutant peptides. In addition, 6-HBs with positively charged substitutions (I37T/N34K, Q41K, or N43K) tend to migrate relatively slower when compared with those without (Fig. 4A).

To further analyze the interaction between the mutant N peptides with C34 in solution, we quantitatively measured the 6-HB formation in reference to the wild-type by ELISA using a 6-HB-specific mAb 2G8 (31, 40). As shown by native PAGE analysis, all mutant N peptides were able to form 6-HB with C34 (Fig. 4B). However, diminished capacity and reduced effi-
ciency in forming 6-HB were found for all mutant N peptides, notwithstanding the substantial degree of variations that were found. As shown in Fig. 4B, N mutant peptides with substitution I37T, N43K, or I37T/N43K are able to form more than 50% of 6-HB compared with the wild-type N36. The remaining N peptide with substitutions, such as Q41K, V38A, and I37T/V38A, severely impaired the 6-HB formation. Furthermore, by comparing the percentage of 6-HB formation with the infectivity for each mutant virus (Fig. 3), a solid positive correlation was found, except for I37T substitution. These results indicate that mutant viruses carrying the key resistant substitutions can still form 6-HB, but with decreased capability. Such decreased capability further led to compromises in their infectivity, a price they had to pay in order to escape efficiently from sifuvirtide inhibition.

Biophysical Characterization of 6-HB Mutants—To study the potential mechanism of observed substitutions on conferring resistance and reduction in infectivity, we have characterized biophysical properties of the key mutants in the context of 6-HBs. We first measured whether these substitutions resulted in any changes in 6-HB with the wild-type N36. The remaining N peptide with substitutions, such as Q41K, V38A, and I37T/V38A, severely impaired the 6-HB formation. Furthermore, by comparing the percentage of 6-HB formation with the infectivity for each mutant virus (Fig. 3), a solid positive correlation was found, except for I37T substitution. These results indicate that mutant viruses carrying the key resistant substitutions can still form 6-HB, but with decreased capability. Such decreased capability further led to compromises in their infectivity, a price they had to pay in order to escape efficiently from sifuvirtide inhibition.

FIGURE 4. Determination of the 6-HB formation between C34 and N36 or a mutated N36 peptide. Wild-type N36 or a mutated N36 peptide was mixed with C34 and subjected to a native PAGE (A) or an ELISA analysis with a 6-HB-specific mAb, 2G8 (B). Error bars, S.D.
tides studied, its reduction in $T_m$ is about 13.31%, which is at a medium level compared with other mutant peptides. Taken together, these findings suggest that thermal stability of 6-HB is probably determined by more factors than $\alpha$-helical content alone. No straightforward correlation between $T_m$ and the level of resistance against sifuvirtide was identified. Therefore, the key substitutions in each mutant virus must be regarded independently in terms of their biophysical as well as biological consequences in contributing to the overall resistant phenotype.

**Structural Analysis of Key Resistant Substitutions**—To further explore the potential mechanism in resistance, we have performed structural analysis of the key resistant substitutions (I37T, V38A, Q41K, Q41H, Q41R, N43K, and N126K) in the context of 6-HB. The locations of these substitutions are indicated in a helical wheel diagram depicting the interacting residues within the trimeric coiled-coil of N-helices as well as those between the N- and C-helix (Fig. 6A). The same substitutions were also superimposed onto a trimeric structure recently obtained from HIV-1 HXB2 (Protein Data Bank code 2X7R) (34) (Fig. 6B). As shown in Fig. 6A, substitutions I37T, Q41K, Q41H, and Q41R are located at position $d$ or $a$, whereas V38A is at position $e$ on the N-helical wheel. Residues at positions $d$ and $a$ are crucial for the formation of trimeric N-helices, whereas those at position $e$ are the key residues mediating interaction between the N- and C-helix (2, 3). Substitution N43K, however, is located at position $c$ on the N-helical wheel, which does not seem to be involved in direct

**FIGURE 5. Biophysical characterization of complexes formed between C34 and N36 or a mutated N36 peptide by CD spectroscopy.** A, CD spectra of wild-type N36 and C34 alone or complexes between C34 and N36 or a mutated N36 peptide. A double minimum at 208 and 222 nm was observed for all complexes formed by the wild type or mutant peptides. B, thermostability of the complexes formed between C34 and a wild-type or mutant N peptide. The unfolding temperature of each complex was scanned at 222 nm by CD spectroscopy, and their $T_m$ values were calculated.
interaction between the N- and C-helix (Fig. 6A). It is therefore possible that reduction in the number of contacts between N-helices or between the N- and C-helix acts as one potential mechanism underlying the ability of these substitutions to confer a resistant phenotype. However, location per se does not seem to be sufficient to account for the resistant phenotype. For example, Q41K and Q41R are highly resistant, whereas Q41H is rather minimal (Table 2). Structural modeling demonstrated that Q41K, Q41H, and Q41R substitutions resulted in increases in both positive charges and the length of the side chains (Fig. 6B). The side chain of the Lys or Arg residue, for example, has extended beyond that of Gln, leading to much closer proximity to the isoleucine (Ile37) or leucine (Leu44) residue on the neighboring N-helix (Fig. 6B). It is possible that such an extension could directly affect the proper formation of trimeric N-helices and then, in turn, indirectly affect the binding with sifuvirtide. In contrast, the side chain of the His residue pointed to the center of the space formed by the trimeric N-helices, which is perhaps large enough to accommodate the extended imidazole group (Fig. 6B). In the case of substitution at position 41, steric orientation seems to play a more important role in viral resistance than the charge properties. Significant reductions in helical content, 6-HB, as well as infectivity of mutant viruses bearing either Q41K or Q41R, but not with Q41H substitution, support this hypothesis (see above).

Mutant viruses with resistant substitutions I37T, V38A, and N43K have distinct structural features, suggesting different underlying mechanisms in conferring resistance. Substitution I37T, shared by two mutant viruses, is located at position d on the N-helical wheel critical for mediating interaction with the neighboring N-helix for the formation of trimeric N-helices (Fig. 6A). Substitution N43K, although not involved in direct interaction with the C-helix, has extended its side chain toward the interacting C-helix and also changed from a polar to a positively charged residue. Such a change may result in enhanced electrostatic attraction with the glutamic acid residue located on the interacting C-helix and, together with I37T, may therefore alter the overall structure of trimeric N-helices in a way that resists binding by sifuvirtide. Substitution V38A, on the other hand, is directly involved in interaction with the C-helix and is therefore expected to have a more dramatic impact on the overall structure of trimeric N-helices, particularly in combination with substitution I37T (Fig. 6A). Significant reductions in helical content, 6-HB, as well as the infectivity of mutant viruses bearing the I37T/V38A double substitution, support this hypothesis (Figs. 3 and 4). In contrast, I37T/N43K double substitution has a quite limited impact on the physical properties of trimeric N-helix and maintains relatively good infectivity (Figs. 3 and 4). Last, substitution N126K is located at position c on the C-helical wheel, which is not involved in the direct interaction with the N-helix (Fig. 6A). However, an additional N126K substitution in the context of I37T/N43K can clearly increase the level of resistance (Table 2) without affecting infectivity (Fig. 3), suggesting its beneficial contribution to the virus-resistant phenotype. The actual mechanism of action of this substitution is quite difficult to predict at this time, although it may help to stabilize the helical structure by forming the intrahelical salt bridge with glutamic acid at position 123 (36, 37).

**DISCUSSION**

In this report, we have shown that *in vitro* selection of HIV-1 with increasing concentrations of sifuvirtide led to several specific mutations in the gp41 region that had not been previously reported. As demonstrated by site-directed mutagenesis, the mutant viruses bearing single substitution Q41K, Q41R, or N43K and the double substitution I37T/N43K or I37T/V38A showed significant increases in resistance against sifuvirtide. Additional N126K substitution can also strengthen the resistance phenotype. Although some mutant viruses with Q41K, Q41R, N43K, or I37T/V38A showed comparable increases in cross-resistance to enfuvirtide, the mutant virus with I37T/N43K was preferentially more resis-

**FIGURE 6.** A, a helical wheel diagram of 6-HB showing the hydrophobic heptad repeat and interaction sites between the mutant N36 and C34. Hydrophobic heptad repeat in both N36 and C34 is indicated by sequential residues located from position a to g. The residues at the a and d positions in N36 peptide are critical for forming the homologous trimeric coiled-coil of NHR, whereas those at the e and g positions in N36 are responsible for interactions with a and d positions in the C34 peptide. Mutated residues (positions 37, 38, and 41) in the N36 peptide mediating the interactions either among N36 peptides or between N36 and C34 peptides are highlighted in black circles, whereas those located at the non-interactive sites are marked in light gray (position 43 and 126). B, structural modeling of key mutated residues Q41R, Q41K, and Q41H in the N peptides and their potential effect on the overall structure of 6-HB. This modeling is based on the trimeric structure recently obtained from HIV-1 HXB2 (Protein Data Bank entry 2X7R), which contains both fusion peptide and membrane-proximal external regions of gp41 (34). Three mutated residues, Lys (orange), Arg (red), and His (yellow), at position 41 were shown to indicate their differences in structure and orientation compared with wild type residue Q (green), which may result in their significant differences in resistance profile against Sifuvirtide and T20 as well as in relative infectivity.
tions in Q41K, Q41R, and I37T/V38A resulted in significant reduc-
tion in α-helical contact as well as 6-HB content, correlating with their severe reduction in infectivity (see above).

Many studies on enfuvirtide-resistant viruses have identified more than one substitution or combinations thereof responsible for conferring the resistant phenotype (36, 37, 41–48). Because most of the substitutions located in the NHR region are critical for binding with enfuvirtide, it was initially hypothesized that these substitutions may exert their impact through reduced direct binding with enfuvirtide. However, subsequent studies demonstrated that different mechanisms may exist for different substitutions in conferring the resistant phenotype. Systemic analysis of substitutions at position 38 has led to the hypothesis that resistance to enfuvirtide could result from the mechanisms of 1) reduced contact, 2) steric obstruction, 3) electronic repulsion, or 4) electronic attraction (48). Our studies of viruses resistant to sifuvirtide agree and go beyond this hypothesis in that not only substitutions affecting N- and C-helix interaction, but also the proper formation of trimeric N-helices, can result in a resistant phenotype. In particular, because many of the key resistant substitutions to sifuvirtide did not involve residues directly interacting between NHR and CHR (see above), the mechanism of reduced contact between these two helices is unlikely to be a major determinant for increased resistance. Substitutions Q41K and Q41R, for example, are located on the inner surface of trimeric N-helices, which predictably would not be able to interact with sifuvirtide. It is possible that extension and orientation of the side chain of the Lys or Arg residue could directly affect the proper formation of trimeric N-helices and then, in turn, indirectly reduce the binding with sifuvirtide. This type of substitution could therefore be classified as a novel mechanism by which the resistant phenotype was achieved through affecting the proper formation of trimeric N-helices. This hypothesis is consistent with an earlier report where N peptide designed to disrupt the internal trimeric coiled-coil of gp41 demonstrated a strong inhibitory effect on HIV-1 (43). The substitution I37T/V38A, on the other hand, is located at positions that directly affect interaction among the N-helices as well as between the N- and C-helix, a type of combination rarely reported previously. Substitution I37T/N43K, however, is expected to have a relatively smaller disruptive effect because substitution N43K is not located at an interactive site with the C-helix. Nevertheless, this kind of substitution is expected to destabilize the overall structure of 6-HB, which can then further lead to a significant reduction in sifuvirtide binding. This type of substitution could then be regarded as another resistant mechanism that is more dependent on the overall structure of the fusion intermediate than reduced direct binding with sifuvirtide. The results in our report are consistent with this hypothesis, where significant reduction in 6-HB content was found for those mutant viruses (see above). Furthermore, resistant mechanisms proposed by Eggink et al. (48) for enfuvirtide could also be applied to some of the substitutions against sifuvirtide. Substitution V38A is likely to fit the proposed group of “reduced contact,” whereas substitution N43K fits the “steric obstruction” group. To some extent, substitution Q41K or Q41R can be classified as belonging to the group of “electrostatic repulsion.” However, as indicated above, the charge property alone is unlikely to be responsible for the observed resistant phenotype because Q41H showed minimal resistance, despite comparable charge property. These findings suggest that the mechanism of resistance is a complex manifestation governed not only by the type of residues but also by their biophysical properties and functionality in the context of virus replication.

Last, now that phase I and II clinical trials of sifuvirtide have been completed, it would be interesting to study resistant substitutions in patients who failed sifuvirtide treatment. Based on resistant mutants and their replication capacity observed in vitro, we hypothesize that single substitution at position 41 (Q41K or Q41R) and double substitution I37T/N43K or I37T/V38A in combination with N126K would emerge in treated patients. However, the mutant virus with double substitution I37T/N43K is likely to dominate because it has demonstrated not only high levels of resistance to sifuvirtide but also minimum disruption in 6-HB formation and in replication activity. We are in the process of verifying our hypothesis, and hopefully in the near future, we will be in a better position to understand the resistant patterns in vivo and their pathogenic potential of mutant viruses in patients with treatment failure.

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