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# Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased sensitivity to an inhibitor of the viral protease

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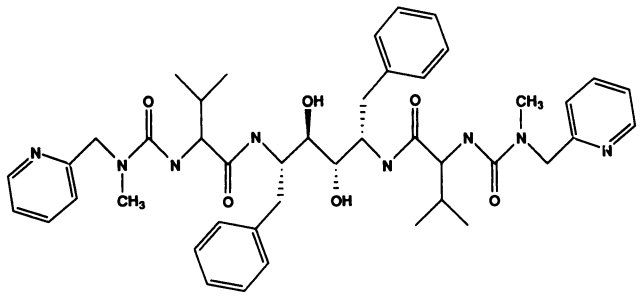
**ABSTRACT** **Inhibitors of the human immunodeficiency virus type 1 (HIV-1) protease represent a promising addition to the available agents used to inhibit virus replication in a therapeutic setting. HIV-1 is capable of generating phenotypic variants in the face of a variety of selective pressures. The potential to generate variants with reduced sensitivity to a protease inhibitor was examined by selecting for virus growth in cell culture in the presence of the protease inhibitor A-77003. Virus variants grew out in the presence of the inhibitor, and these variants encoded proteases with reduced sensitivity to the inhibitor. Variants were identified that encoded changes in each of the three subsites of the protease that interact with the inhibitor. HIV-1 displays significant potential for altering its interaction with this protease inhibitor, suggesting the need for multiple protease inhibitors with varying specificities.**

All retroviruses encode a protease that cleaves the polyprotein precursors Gag and Gag/Pol during virus assembly (reviewed in ref. 1). If protease activity is blocked, then noninfectious particles are formed, which are composed of unprocessed precursors (2–4). The viral protease is therefore an important target for drug development. Numerous compounds that are potent inhibitors of the protease of human immunodeficiency virus type 1 (HIV-1) and that effectively inhibit virus replication (5–8) have been developed. The structure-based design of the C2 symmetric inhibitors has resulted in a distinct class of antiviral compounds, several of which exhibit acceptable pharmacologic properties. One such compound, A-77003 (9), is shown below:




The retroviral protease must interact with a variety of distinct protein sequences that together constitute the processing sites in the Gag and Gag/Pol precursors (reviewed in ref. 1). Substitutions of the amino acids that make up the protease subsites, the regions of the protease that interact with the side chains of the amino acids at the cleavage sites, can give rise to proteases with altered specificity (10–12). Thus it appears that the protease has sufficient flexibility in its sequence requirements to permit sequence variability that could affect interaction with a protease inhibitor. An initial demonstration of this possibility was shown by the work of Otto *et al.* (13), where selection for HIV-1 replication in the presence of an inhibitor related to A-77003 resulted in the outgrowth of a variant with a 6- to 8-fold change in the sensitivity of the virus to the inhibitor.

Our overall strategy was to use cell culture systems to grow HIV-1 in the presence of the inhibitor A-77003 and then test the resulting virus for sensitivity to the inhibitor. In addition, we have characterized the coding domain of the viral protease in cultures that had undergone extensive selection. Both analyses indicate that HIV-1 is capable of generating variants with reduced sensitivity to the inhibitor, and characterization of the mutant proteases demonstrates that multiple residues can be changed to alter the interaction between the protease and the inhibitor.

## METHODS

**Selection of Virus Variants.** CEM cells were acutely infected (14) with a virus derived from the HXB2 molecular clone of HIV-1 (15). After 4 hr the cells were placed in a total volume of 19 ml of medium [RPMI 1640 with 2 mM glutamine and 10% (vol/vol) fetal calf serum]. The medium was replaced every 2 days, and the cells were passed at a split ratio of 1:2 as needed (every 2–3 days). The concentration of A-77003 used in the initial round of infection was 0.1 $\mu$M. After the infection had spread through the culture (i.e., all of the cells were involved in syncytia), the cells were pelleted and the supernatant was used to start the next round of selection. Cultures were grown in the presence of the previous concentration of inhibitor and 0.1, 0.2, and 0.3 $\mu$M higher concentrations of A-77003. The supernatant from the culture that produced syncytia in the presence of the highest concentration of inhibitor was used to initiate the next round of infection.

In the selection using chronically infected cells, CEM cells chronically infected with the HIV-$1_{LAI}$ were placed in fresh medium every 2–3 days, with the inhibitor concentration maintained at a constant level. When the medium was replaced, the cells were adjusted to a concentration of $5 \times 10^5$

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Abbreviation: HIV-1, human immunodeficiency virus type 1.

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cells per ml. The chronically infected cell line was established as the outgrowth of CEM cells acutely infected with HIV-$1_{LAI}$. The cells were passaged ≈40 times prior to selection with the inhibitor.

**A-77003 Sensitivity Testing.** Virus titer was determined using the assay of Kimpton and Emerman (16) or by end-point dilution using CEM cells. To test for drug sensitivity, CEM cells were infected with the indicated virus stock by placing $2 \times 10^6$ cells and $2 \times 10^5$ infectious units of each virus in a total of 1.0 ml of tissue culture medium for 5 hr at 37°C. The total volume of medium was then increased to 19 ml. A-77003 was added to each flask to the indicated concentration. After 3 days, and each day thereafter, the cells in each flask were recovered by centrifugation and resuspended in fresh medium, and the appropriate amount of A-77003 was added. The sensitivity of the different virus stocks to A-77003 was estimated by comparing the concentration of drug needed to reduce the production of viral p24 capsid antigen in the cell-free supernatant by 50% (inhibitory concentration 50%, or $IC_{50}$).

**Recovery and Amplification of Protease Coding Domain Sequences from Infected Cells.** Genomic DNA was purified from ≈$5 \times 10^6$ infected cells by lysis [proteinase K (500 μg/ml)/0.5% SDS/10 mM Tris, pH 7.5/30 mM NaCl/20 mM EDTA, 55°C, 2 hr], followed by extraction with phenol/chloroform and ethanol precipitation. Two rounds of nested-primer PCR were used to amplify a DNA product containing the protease coding domain. First round conditions were 2 μM each outer primer, 400 μM of each dNTP, 50 mM KCl, 10 mM Tris (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 5 units of *Taq* polymerase (Promega); cycle 1: 94°C for 5 min, 50°C for 60 sec, and 72°C for 90 sec; cycles 2–29: 94°C for 30 sec, 50°C for 60 sec, and 72°C for 90 sec; cycle 30: 94°C for 30 sec, 50°C for 60 sec, and 72°C for 10 min. Second round reactions used the first round product as template and an interior set of primers but were otherwise identical to first round conditions. The primer sequences are as follows: primer 1, 5′-GGAATATTGCTGGTGATCCTTTCCATCCC-3′; primer 2, 5′-CTGTTGGAAATGTGGAAAGGAAGGACACC-3′; primer 3, 5′-GCACATTGTACTGATATCTAATCCC-3′; primer 4, 5′-TTAGGGAAGATCTGGCCTTCCTAC-3′.

PCR products were digested with *Eco*RV and *Bgl* II and cloned into pIBI20. Individual clones were isolated in *Escherichia coli* strain DH5αFIQ and sequenced using a dideoxy chain-termination protocol for either double-stranded or single-stranded templates (United States Biochemical).

**Characterization of Mutant Proteases.** Mutant protease coding regions were inserted into a T7-based plasmid (17) and expressed in *E. coli*. Protease was purified from the inclusion body fraction by a modification of the procedure of Cheng *et al.* (18) as modified by M. Moody and R.S. (unpublished results). Protease activity was assayed using a modification of a radiometric assay described by Ido *et al.* (19). Reactions were performed at 30°C in 25 μl with 0.2 M NaCl, 1 mM dithiothreitol, 0.1% Triton X-100, and 100 mM sodium acetate (pH 5.5). The substrate [$^3$H]Ac-SQNYPIVR had a specific activity of 11 mCi/mmol (1 Ci = 37 GBq) and was present at a concentration of 0.5 mM. Reactions were linear over the 30-min period of incubation with <10% substrate cleavage and were terminated by the addition of Dowex X-50W-X4 (5% wt/vol) in 0.1 M acetic acid. The resulting supernatant contained only the acetylated cleavage product, which was quantitated by liquid scintillation counting. Apparent $K_i$ values were determined as described (20), using a nonlinear regression routine (21), which was kindly provided by D. Northrop (University of Wisconsin).

## RESULTS

***In Vitro* Selection of Less Susceptible Variants in Acutely Infected Cells.** Two approaches were used to select for virus with reduced sensitivity to the inhibitor. In the first scheme, virus was passaged in the presence of increasing concentrations of A-77003. For the initial round of selection, CEM cells were infected with virus in the presence of 0.1 μM of the inhibitor (slightly above the $IC_{50}$). After nine passages in the presence of an escalating inhibitor concentration, the infection proceeded in the presence of an inhibitor concentration of 0.9 μM.

Virus harvested at the end of passages 4 and 8 was assayed for sensitivity to A-77003. Virus passaged nine times without inhibitor remained sensitive to the inhibitor (Fig. 1*A*). A change in sensitivity was observed, however, with the virus passaged in the presence of inhibitor (Fig. 1*B*). We estimate there is an ≈10-fold increase in the $IC_{50}$ of the virus produced by passage in the presence of inhibitor. Virus obtained after four passages in the presence of inhibitor was indistinguishable in its sensitivity compared to the parental virus (data not shown).

We examined the protease sequence for the presence of mutations using the PCR to amplify the complete protease coding domain. The amplified viral DNA was cloned in a phagemid vector, and individual clones were sequenced until the extent of sequence heterogeneity within the population could be assessed. A comparison of the sequences from the parent virus with those obtained from the passage 6 virus demonstrates that several changes accumulated during the selection period (Fig. 2*A*). Encoded amino acid changes that appeared more than twice in 40 clones are valine at position 32 replaced by isoleucine (V32I), valine at position 82 replaced by isoleucine (V82I), and methionine at position 46 replaced by leucine. The pattern of substitutions became

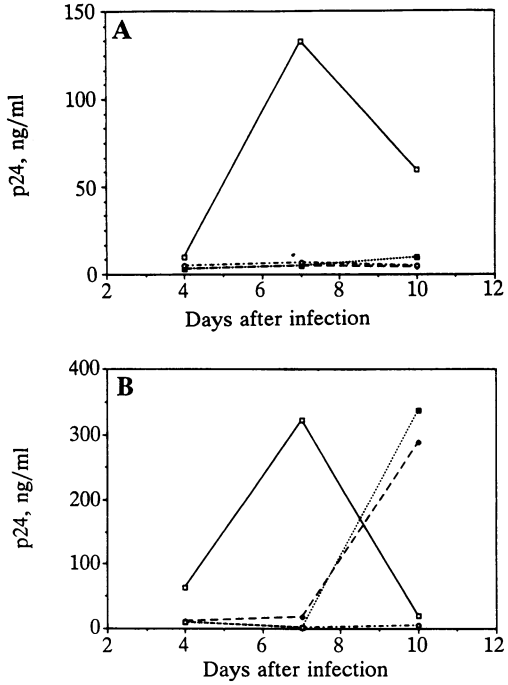


FIG. 1. HIV-1 passaged in the presence of the symmetric protease inhibitor A-77003 displays reduced sensitivity. Sensitivity testing was performed on virus stocks generated by passage in the absence (*A*) or presence (*B*) of increasing concentrations of A-77003. Virus growth was monitored in the absence of inhibitor (□) or in the presence of 0.1 μM (●), 1.0 μM (■), or 1.5 μM (○) A-77003. The virus stocks used for these experiments were generated by the transfection of HeLa cells with the HXB2 infectious clone (15) using the calcium phosphate method (22).

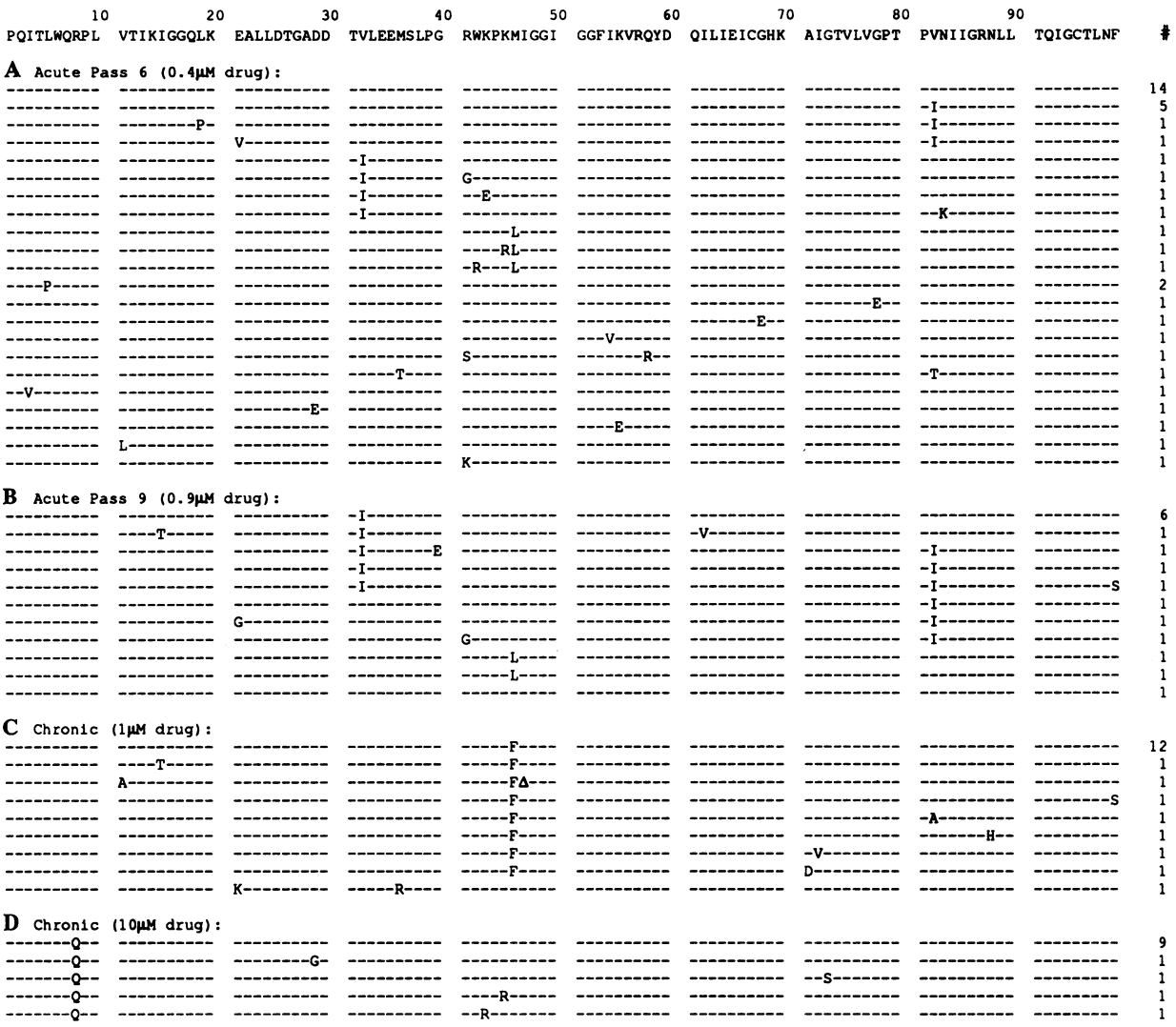


FIG. 2. Deduced amino acid sequences of HIV-1 protease domains amplified from HIV-1-infected cells cultured in the presence of A-77003. Virus was grown either by passage of culture supernatants into fresh cultures with increasing concentrations of inhibitor (acute infection) or by passage of infected cells at constant inhibitor concentration (chronic infection). (*A*) Sequences derived from the sixth passage of an acutely infected culture. The A-77003 concentration in the culture was 0.4 μM at this point. (*B*) Sequences derived from the ninth passage of an acutely infected culture. The A-77003 concentration in the culture was 0.9 μM at this point. (*C*) Sequences derived from chronically infected cells after 3 months of culture in the presence of 1.0 μM A-77003. (*D*) Sequences derived from chronically infected cells after 3 months of culture in the presence of 10 μM A-77003.

more distinctive by passage 9. At this point, 10 of 16 clones had the valine to isoleucine change at position 32, and 6 of 16 clones had the valine to isoleucine change at position 82. Furthermore, 3 of these 16 clones were substituted at both positions 32 and 82 to encode isoleucine (V32I/V82I) (Fig. 2*B*). Two of the 16 clones had leucine substituted for methionine at position 46. An examination of the viral sequences derived from virus passaged in the absence of selection revealed six unrelated substitutions in 23 clones sequenced (data not shown).

***In Vitro* Selection of Less Susceptible Variants in Chronically Infected Cells.** The second selection scheme took advantage of the continued replication of HIV-1 in cells chronically infected with the virus (23). The chronically infected cells were grown in the presence of either 1.0 μM or 10 μM of A-77003 for 3 months. In the presence of 10 μM of A-77003, supernatant p24 antigen declined by ≈100-fold within the first 2 weeks of culture and remained relatively constant. Only modest declines were observed in the presence of the inhibitor at 1 μM. Supernatant infectivity in the presence of either 1 μM or 10 μM A-77003 declined to zero after only 1 week. However, at the end of the 3-month period, infectivity, albeit low, was again observed in the supernatant of these cultures, suggesting that selection had given rise to a less sensitive virus population (data not shown).

We amplified proviral DNA from the chronically infected cells selected for 3 months at the two drug concentrations. Of the clones isolated from the culture selected with 1.0 μM

Table 1. Apparent $K_i$ values for A-77003

| Protease | Apparent $K_i$, nM |
|---|---|
| WT | 0.5 ± 0.1 |
| V82I | 0.5 ± 0.1 |
| M46L | 1.3 ± 0.2 |
| M46F | 2.0 ± 0.5 |
| V32I | 3.8 ± 0.7 |
| V32I/V82I | 11 ± 1 |
| R8Q | 31 ± 9 |

WT, wild type. In the designation of mutations, the wild-type amino acid precedes the HIV-1 protease residue number, followed by the substituted residue using the single-letter amino acid abbreviations.

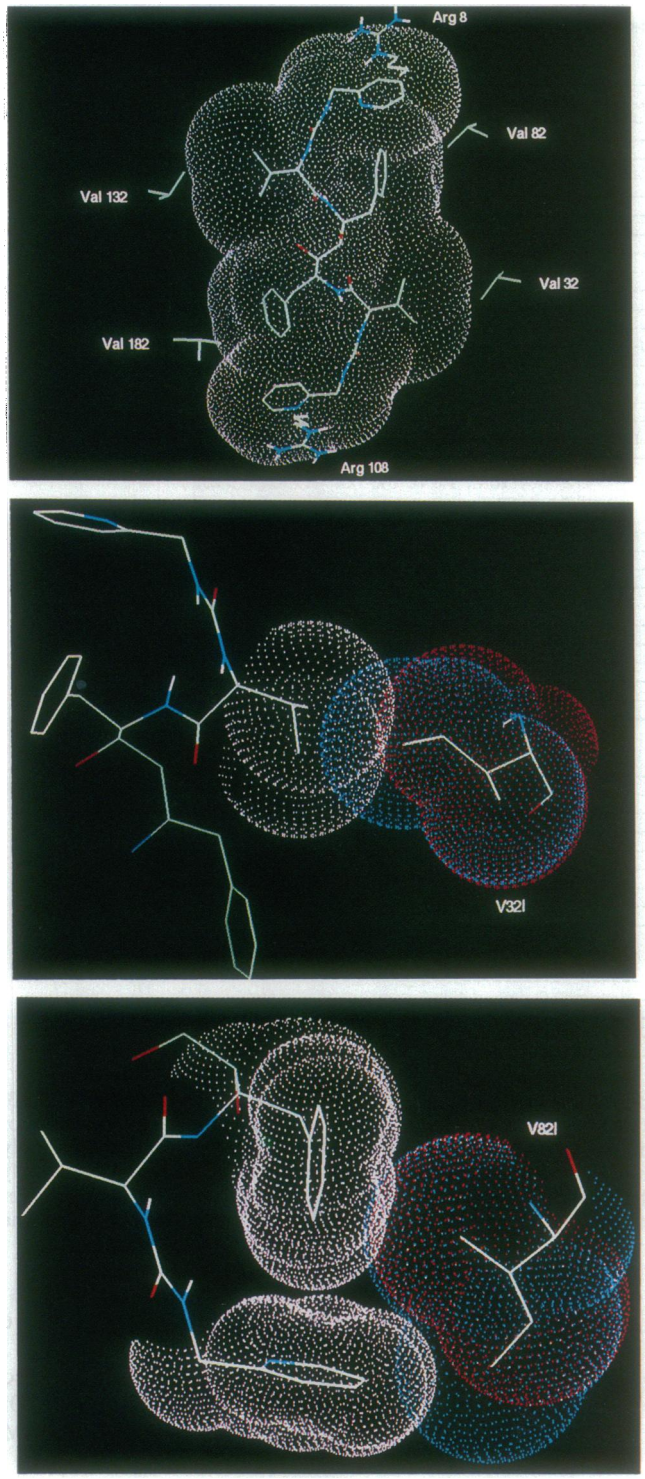


FIG. 3. Modeling of altered interactions between A-77003 and mutant HIV-1 proteases. (*Top*) The structure of A-77003 and its van der Waals surface are shown with the positions of selected side chains of the wild-type HIV-1 protease (24). The side chains shown are Arg8, Val32, and Val82; equivalent side chains from the second subunit of the protease dimer are also shown (Arg108, Val132, and Val182). (*Middle*) The van der Waals surface of the inhibitor isopropyl side chain occupying the P2 position is shown (white) with the van der Waals surface of the wild-type Val32 side chain (red) and the substituted Ile32 side chain and its van der Waals surface (blue). (*Bottom*) The structure and van der Waals surface of the inhibitor·P1 benzyl group and P3 pyridyl group are shown (white). Also shown are the van der Waals surface of the wild-type Val82 side chain of the protease (red) as well as the mutant Ile82 side chain and its van der Waals surface (blue). Modeling was done by addition of a methylene group to valine and adjustment to give a conformation typical of isoleucine. It was possible to rotate the V82I modeled structure to minimize close contacts, while the V32I modeled structure had unfavorable contacts in all orientations examined. No minimization was done to adjust the positions of other side chains in the modeled structures.

A-77003, 18 of 19 encoded phenylalanine instead of methionine at position 46 (M46F; Fig. 2*C*). Of the clones isolated from the culture selected with 10 $\mu$M A-77003, all 13 encoded glutamine instead of arginine at position 8 (R8Q; Fig. 2*D*). Sequences obtained from 12 clones recovered from chronically infected cells passaged in the absence of inhibitor showed minor variations unrelated to the changes seen after selection (data not shown).

**Susceptibility of the Mutant Enzymes to A-77003.** Viral protease coding domains coding the substitutions R8Q, V32I, V32I/V82I, M46L, M46F, and V82I were expressed in *E. coli*. The protease variants were purified and individually tested for sensitivity to A-77003 (Table 1). The apparent $K_i$ for the V82I mutant was no different from that obtained using wild-type protease. However, the V32I mutant and the R8Q mutant were ≈7- and 60-fold less sensitive to inhibition by A-77003, respectively. The double mutant V32I/V82I had an apparent $K_i$ that was ≈20-fold greater than wild-type protease. A leucine or phenylalanine mutation at position 46 yielded an enzyme that was ≈2- to 4-fold less sensitive to A-77003 inhibition compared to the wild-type protease.

**Evaluation of the Structural Basis for the Reduced Drug Sensitivity.** The structural basis for the reduced drug sensitivity of the various protease mutants was evaluated with reference to the crystal structure of the A-77003/HIV-1 protease complex (24). The wild-type residues V82, V32, and R8 make symmetry-related side chain contacts with the inhibitor in the S1/S1′, S2/S2′, and S3/S3′ subsites, respectively (Fig. 3 *Top*). M46 is on the protease flap, and its side chain extends into the solvent.

We modeled the V32I and V82I mutations into the crystal structure of the A-77003/HIV-1 protease complex (Fig. 3 *Middle* and *Bottom*). Both substitutions lead to repulsive van der Waals interactions due to steric overlap. In the case of V32I, the isoleucine residue is tightly packed and has little room for adjustment. The inhibitor may still bind, but with lower affinity, presumably due to a repositioning of the isopropyl P2 and P2′ side chains of the inhibitor. In the V82I mutant, the isoleucine side chain has more freedom of movement within the S1/S1′ subsites to help alleviate repulsive contacts with the P1/P1′ benzyl groups of A-77003. The synergistic effects of V82I and V32I may be explained by the fact that these two bulky substituents closely impinge on opposite sides of the inhibitor in a pincer-like fashion (Fig. 3 *Top*).

The protease variant with the largest increase in the apparent $K_i$, R8Q, was shown to result in decreased van der Waals and charge-induced dipole interactions between the enzyme guanidinium and inhibitor pyridine groups (25). The selection of mutations at M46 is more difficult to rationalize on structural grounds since this residue does not play a direct role in binding to A-77003.

## DISCUSSION

Inhibitors of the protease of HIV-1 have significant potential as therapeutic agents in the treatment of AIDS. One concern in the use of any drug is the possibility that resistant virus variants might arise during therapy, as has been seen with inhibitors to the HIV-1 reverse transcriptase (26). We have evaluated the growth of HIV-1 in the presence of a small molecule protease inhibitor and determined that virus populations with reduced sensitivity may develop during selection in cell culture. An analysis of the evolution of protease

coding domain sequences indicates that specific substitutions, which represent minority subpopulations early in selection, ultimately predominate in the culture. The amino acid substitutions are predicted to result in unfavorable interactions between the inhibitor and the enzyme.

HIV-1 with the protease substitution R8Q is attenuated for growth (ref. 25; unpublished observation). Thus, the appearance of this mutation, which was also selected by Ho *et al.* (25) using A-77003, demonstrates the strong antiviral effect of A-77003, which can lead to the selection of a variant with reduced replicative capacity. A virus with the protease double substitution V32I/V82I grows well as a virus and has a 5- to 10-fold increase in its $IC_{50}$ (unpublished observation). Otto *et al.* (13) have recently shown that selection with a related C2 symmetric inhibitor resulted in the appearance of an HIV-1 variant with a V82A substitution and that this variant had a 6- to 8-fold reduction in sensitivity to this compound. One example of this substitution was seen in our selection of chronically infected cells at 1 $\mu$M inhibitor concentration (Fig. 2*C*); in this case, the V82A substitution occurred with the M46F substitution in the flap. A substitution at position 84 (I84V) has also been detected after selection with another protease inhibitor (27).

Our analysis of the virus variants encoding proteases with reduced sensitivity to A-77003 indicates that all three subsites at which this inhibitor contacts the protease can be mutated. This suggests that the virus has significant flexibility to alter the interaction between the protease and the inhibitor and still maintain the functional properties of the protease required for virus replication. Knowledge of the changes that alter the sensitivity of HIV-1 to A-77003 provides a data base with which to evaluate sequence heterogeneity in patients undergoing therapy with this type of inhibitor. In addition, the mutant proteases represent important reagents for testing other classes of protease inhibitors for cross-resistance. Finally, an understanding of the mechanism of altered sensitivity provides an opportunity for the design and testing of new inhibitors that can take advantage of the sequence variants likely to arise after initial treatment.

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