Characterization of Human Immunodeficiency Virus Type 1 Variants with Increased Resistance to a C₂-Symmetric Protease Inhibitor

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Inhibitors of the human immunodeficiency virus type 1 protease represent a promising class of antiviral drugs for the treatment of AIDS, and several are now in clinical trials. Here, we report the in vitro selection of viral variants with decreased sensitivity to a C₂-symmetric protease inhibitor (A-77003). We show that a single amino acid substitution (Arg to Gln or Lys) at position 8 of the protease results in a substantial decrease in the inhibitory activity of the drug on the enzyme and a comparable increase in viral resistance. These findings, when analyzed by using the three-dimensional structure of the protease-drug complex, provide a strategic guide for the future development of inhibitors of the human immunodeficiency virus type 1 protease.

Several antiviral agents, targeting the reverse transcriptase of the human immunodeficiency virus type 1 (HIV-1), have been developed for the treatment of AIDS. Since these drugs have limited clinical benefit due in part to the development of viral resistance (7, 10, 11, 15–17, 22, 24, 25, 27), inhibitors against other HIV-1 targets are urgently needed. The HIV-1 aspartic protease is a logical target for rational drug screening and design (5). This viral enzyme functions as a homodimer in which the two subunits are related by a twofold (C₂) axis of symmetry (14, 20, 29). A single enzyme active site is formed at the interface between the subunits. Posttranslational processing of Gag and Pol polypeptides by the protease is required for viral maturation and infectivity. To date, numerous compounds that block protease function and inhibit HIV-1 replication in vitro have been identified (4, 6, 12, 13, 18, 21, 26, 28). Recently, Erickson and coworkers (6, 12, 13, 21) reported a series of inhibitors designed to complement the unique C₂-symmetric architecture of the enzyme active site. Their anti-HIV-1 activities in vitro have been well documented, with 50 and 90% inhibitory doses (ID₅₀ and ID₉₀) in the range of 0.1 to 1.0 μM (12, 13, 21). Several of these compounds have shown favorable pharmacokinetic and toxicity profiles in animals, including A-77003, which has entered into phase 1 studies with HIV-1-infected individuals.

We began to examine HIV-1 resistance to A-77003 by growing NL4-3 (1), a virus derived from an infectious molecular clone (multiplicity of infection, 0.01 50% tissue culture infective dose per cell), in MT-4 cells (2 × 10⁵/ml) in the presence of a subinhibitory concentration (0.02 μM) of A-77003. When viral replication (supernatant p24 antigen) was evident, the supernatant fluid was harvested and used to infect additional MT-4 cells in the presence of a higher concentration of A-77003. As summarized in Table 1, this selection scheme was serially carried out for 19 passages using drug concentra-

tions of up to 10 μM. The cell pellet from each passage was stored at –80°C for sequencing studies. Viral populations from passages 4, 14, 17, and 19 along with the parental NL4-3 were each propagated, their titers were determined, and the populations were tested for sensitivity to A-77003. A-77003 sensitivity studies were performed with MT-4 cells and 500 50% tissue culture infective doses of each virus, and the inhibitory effect of the drug was assessed by measuring p24 antigen in the culture supernatant on day 4. As shown in Table 1, a fourfold increase in ID₅₀ was seen for the passage 14 virus (P14), while a 35-fold increase was observed for the passage 19 virus (P19), together with a substantial increase in ID₉₀.

P14 and P19 were further characterized to determine the sequence changes responsible for the reduced sensitivity to A-77003. Although it is theoretically possible that alterations in other genomic regions could affect drug susceptibility, we focused on the protease-encoding region of *pol* as the most likely to account for the increased resistance. DNA was extracted from P14 and P19 and subjected to PCR using primers 5'-AGAAGAGAGCTCCAGGTTTGGGGA A-3' (2166 to 2190) and 5'-GGCTTGAATTCTACTGGTA CAGTCT-3' (2566 to 2590). The amplification was carried out in a solution containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 200 μM dATP, dCTP, dGTP, and dTTP each; 0.2 μM primers; 2.5 mM MgCl₂; 2.5 U of *Taq* polymerase; and 1 μg of DNA in 100 μl. The PCR conditions were melting at 94°C, annealing at 50°C, and extending at 72°C; each step was carried out for 30 s in a Perkin-Elmer 9600 Thermocycler for a total of 30 cycles. The PCR products were cleaved with *Eco*RI and *Sac*I and cloned into M13 phage, and nucleotide sequences were determined.

The deduced amino acid sequences in Fig. 1 show that, compared with the parental NL4-3, 9 of 10 M13 clones derived from P14 contained an arginine-to-glutamine substitution at amino acid 8 of protease (R8Q). One clone (P14-10), lacking the R8Q mutation, had a valine-to-alanine substitution at position 82 (V82A) instead. Eight of 10 clones from P14 also contained a methionine-to-isoleucine substitution at position 46 (M46I). Every M13 clone from P19 contained either the

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TABLE 1. In vitro selection of A-77003-resistant variants of HIV-1

Passage no.	A-77003 concn (μ M) in selection	Sensitivity to A-77003 ^a (μ M)	
		ID ₅₀	ID ₉₀
0 ^b		0.2	1.0
1-4	0.02-0.20	0.4	1.0
5-14	0.50-1.50	0.8	1.0
15-17	2.00-5.00	4.0	10.0
18-19	7.00-10.00	7.0	>10.0

^a For virus from the last in the range of passages.^b Parental virus.

same R8Q substitution or a lysine substitution at the same position (R8K); every P19 clone also had the M46I mutation. In addition, several scattered substitutions were found in some clones between amino acids 49 and 80 of the protease (Fig. 1).

The consistent changes at amino acids 8 and 46 suggest that these substitutions could be responsible for the increased resistance to A-77003. Arg-8 is highly conserved among all published sequences for HIV-1, HIV-2, and simian immunodeficiency virus proteases (8, 19). In contrast, Ile-46 was seen in only one of 21 HIV-1 sequences but is found in all HIV-2 and simian immunodeficiency virus sequences (8, 19).

To address whether these observed mutations were responsible for the increased resistance to A-77003, site-directed mutagenesis was performed on the infectious molecular clone pNL4-3. R8Q, R8K, and M46I mutants as well as an R8Q/M46I double mutant were generated and reconstructed back into pNL4-3. A 7,018-bp *SphI*-*Bam*HI fragment of NL4-3 was cloned into a pALT1 (Promega, Madison, Wis.) vector, and the mutagenesis was conducted according to the manufacturer's instructions. The mutagenized fragment was then reconstructed back into pNL4-3, and infectious virus was obtained by electroporation of the clone into MT-4 cells (3). As shown in Fig. 2A, the R8Q mutant replicated less efficiently than the parental NL4-3, whereas the M46I virus grew well. Interestingly, the R8Q/M46I double mutant replicated efficiently, suggesting that the M46I mutation may compensate for the deleterious effect of the R8Q change. Likewise, with an additional base substitution at codon 8, the R8Q variant was further mutated to give rise to an R8K variant, which grew as efficiently as the parental virus (Fig. 2A).

Each of these mutant viruses was propagated, the titers were determined, and the viruses were tested for sensitivity to

A-77003. As shown in Fig. 2B, the M46I mutant and the parental virus were equally sensitive to A-77003. However, the R8Q, R8K, and R8Q/M46I mutants were less sensitive, with approximate ID₅₀ and ID₉₀ of 1.0 to 2.0 and 10 μ M, respectively, suggesting a 10-fold decrease in sensitivity to A-77003. Additional studies to determine whether the R8K/M46I double mutant and the scattered changes between amino acids 49 and 80 contribute to the increased resistance exhibited by P19 are in progress. Nevertheless, the data in Fig. 2B suggest that the R8Q and R8K mutations are each responsible for a substantial portion of the increased resistance to A-77003.

To confirm that the decrease in A-77003 sensitivity was mediated by changes in the inhibitory activity of the drug on the mutant proteases, the enzymes from NL4-3, R8Q, and M46I viruses were individually expressed and purified for determination of the inhibitory constant (K_i) for A-77003. Each HIV-1 mutant protease was overexpressed in insoluble form in *E. coli* after arabinose induction. Following solubilization in 8 M urea, the samples were passed through a DE-52 cellulose (Whatman) column, dialyzed to remove urea, and affinity purified by elution through a Pepstatin A agarose (Pierce) column. Final purification of active fractions was accomplished by elution through an HD/M HPLC Poros column (Perspective Biosystems). Inhibition of HIV-1 protease was measured by using the fluorogenic substrate DAB-CYL-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS as previously described (12, 13, 21). K_i values were calculated by using equations for tight-binding inhibitors (our unpublished data). As shown in Fig. 3, the K_i of NL4-3 protease for A-77003 was found to be 84 μ M. A similar K_i (120 μ M) was found for the M46I mutant protease; however, the K_i for the R8Q mutant protease was 2,700 μ M, an approximately 32-fold increase compared with the parental protease. Therefore, we conclude that the R8Q mutation significantly decreased the protease inhibition by A-77003 whereas the M46I mutation had only a minimal effect. Similar K_i determinations are now in progress for the R8K, R8Q/M46I, and R8K/M46I mutant proteases.

The crystal structure of A-77003 complexed with the wild-type protease shows that the aromatic pyridine groups at the distal ends of the drug interact closely with the arginine residue at position 8 of each subunit of the protease homodimer (Fig. 4, top) (our unpublished data). The planes of the interacting pyridine ring and guanidinium group are approximately parallel and stacked with an interplanar distance of 3.4 to 3.6 Å. Interactions between aromatic and amino groups in proteins have been attributed to weakly polar effects arising from

	20	40	60	80
NL4-3	PQITLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI GGFIXVRQYD QILIEICGKH AIGTVLVGPT FVNILGRNLL TQIGCTLNF			
P14-01	-----Q--	-----I--	-----	-----
P14-02	-----Q--	-----I--	-----	-----
P14-03	-----Q--	-----E--	-----I--	-----R--
P14-04	-----Q--	-----I--	-----	-----
P14-05	-----Q--	-----I--	-----	-----
P14-06	-----Q--	-----I--	-----	-----
P14-07	-----Q--	-----I--	-----	-----
P14-08	-----Q--	-----I--	-----	-----
P14-09	-----Q--	-----I--	-----	-----
P14-10	-----Q--	-----I--	-----	-----A--
P19-28	-----Q--	-----I--	-----G--	-----
P19-54	-----Q--	-----I--	-----P--	-----H--
P19-56	-----Q--	-----I--	-----E--	-----R--
P19-71	-----K--	-----I--	-----P--	-----H--
P19-73	-----Q--	-----I--	-----	-----
P19-76	-----K--	-----I--	-----	-----
P19-78	-----K--	-----I--	-----P--	-----
P19-80	-----Q--	-----I--	-----P--	-----

FIG. 1. Amino acid sequences of proteases from NL4-3 and HIV-1 variants obtained from passages 14 and 19. The sequence of NL4-3 propagated in our laboratory has at position 57 of protease an R instead of the G in the published sequence.

FIG. 2. (A) Replication kinetics of R8Q (○), M46I (△), R8K (▽), and R8Q/M46I (●) mutants and parental NL4-3 (□) in MT-4 cells. Five hundred 50% tissue culture infective doses of each virus were used to inoculate 10⁶ MT-4 cells, and supernatant p24 antigen expression was monitored on days 3, 6, and 9 of culture. (B) Sensitivities of R8Q, M46I, R8K, and R8Q/M46I mutants and parental NL4-3 to A-77003.

charge quadrupole electrostatic interactions, and similar types of interactions have been observed with protein-ligand binding (2). We have modelled the R8Q mutant into the crystal structure of the A-77003–protease complex (Fig. 4, bottom). In this model, Gln-8 makes less extensive van der Waals contacts with the pyridine groups. In addition, the strength of the electrostatic interaction should be reduced owing to the subtractive effects of both the reduced positive charge of the amide side chain of glutamine and the greater distance dependence of the amide dipole-pyridine ring interaction. We be-

lieve that these changes provide a reasonable explanation for the reduced inhibitory activity of A-77003 on the R8Q protease and the consequent A-77003 resistance of our selected viral variants. The Arg-to-Lys substitution at residue 8 represents a conservative change, which perhaps explains why the R8K variant shows no gross defect in replication efficiency. Computer modelling to explain the increased A-77003 resistance of this viral variant is now in progress.

In a separate study, Kaplan and Swanstrom (10a) have found that the identical mutation led to a substantial increase

Inhibitor	Structure	Drug Sensitivity ID ₅₀ (μM)		Drug-Protease Binding K _i (pM)		
		NL4-3	P19	NL4-3	R8Q	M46I
A-77003		0.2	6.0	84	2,700	120
A-76889		0.9	4.7	1,000	23,000	ND
A-76928		0.1	2.8	77	7,200	ND
A-76215		7.0	8.0	22,000	37,000	ND
Ro 31-8959		0.01	0.01	73	270	ND
L-689,502		0.01	0.01	51	140	54

FIG. 3. Sensitivity of P19 and binding kinetics of the R8Q mutant to other protease inhibitors. NL4-3 and P19 were assayed for sensitivity to other inhibitors by using MT-4 cells and 500 50% tissue culture infective doses of virus as described in the text.

FIG. 4. (Top) Interactions between Arg-8 and the position 3 pyridine group of A-77003. Van der Waals surfaces are displayed for Arg-8 (blue), Asp-129 (red), and A-77003 (white). (Bottom) Space-filling views of the wild-type Arg-8 and mutant Gln-8 contacts with A-77003 (yellow). Atoms of the enzyme are color coded by type: nitrogen, blue; oxygen, red; carbon, green. Arg-8 and Gln-8 side chains are on the bottom right side of the complex and form contacts with Asp-129 carboxylate (bottom left) and the position 3 pyridine (middle) groups of the enzyme and inhibitor, respectively.

in the K_i of A-77003 for the mutant protease. They have also found substitutions at amino acids 46 (M46L or M46F) and 82 (V82I), although these changes alone resulted in only minor changes in the K_i . Studying a different C_2 -symmetric inhibitor of protease, Otto et al. (23) have found that the V82A mutation resulted in a six- to eightfold reduction in drug sensitivity of the mutant virus. This mutation was found in one of our P14 clones.

Our structural analysis predicted that the R8Q mutant would show cross-resistance to other C_2 -symmetric inhibitors of similar length with pyridine rings at the distal ends. Indeed, as shown in Fig. 3, cross-resistance was found for two similar compounds (A-76889 and A-76928) tested against P19. In

contrast, a shorter inhibitor (A-76215) and two structurally unrelated, nonsymmetric inhibitors (Ro 31-8959 [4, 26] and L-689,502 [28]) did not show cross-resistance. We also assessed the inhibitory activity of these inhibitors on NL4-3 and R8Q proteases. The results in Fig. 3 show that compounds closely related to A-77003 have lower affinities for the R8Q protease than for the NL4-3 protease. In contrast, for those drugs lacking terminal pyridine rings, the K_i values are approximately equal for the R8Q and NL4-3 proteases. Taken together, these findings suggest that different resistance mutations might be selected by different classes of protease inhibitors. Thus, structurally different protease inhibitors might be useful in combination to slow or prevent the development of resistant mutants.

Arg-8 in HIV-1 protease also plays a role in dimer stability, since it forms a favorable electrostatic interaction with Asp-129 from the other subunit (Fig. 4, top) (14, 20, 29). This symmetric salt bridge is maintained in the A-77003 complex but is abolished in the R8Q mutant, which can at best form a hydrogen bond between Gln-8 and Asp-129. Thus, the inferior growth characteristics of the R8Q mutant in vitro may be a reflection of reduced enzyme stability. The restoration of wild-type replication kinetics for the R8Q/M46I double mutant is difficult to explain on structural grounds alone. Met-46 protrudes from the flap into the solvent and does not make contacts with A-77003 (our unpublished data), but it may play a role in binding the Gag and Pol polypeptide substrates (9). Alternatively, the compensatory effect of the M46I mutation on virus growth may be due to a kinetic effect on protease activity by virtue of its position on the flap, where it may modulate flap dynamics.

In conclusion, we have selected HIV-1 variants in vitro which have increased resistance to a protease inhibitor. Although other mutations may contribute, the R8Q and R8K mutations in the protease appear to be primarily responsible for the decrease in the inhibitory activity of the drug on the enzyme and the consequent increase in viral resistance. These findings are similar to what has been found for HIV-1 resistance to inhibitors of reverse transcriptase, in which a single amino acid substitution resulted in greater drug resistance (7, 10, 11, 15–17, 22, 24, 25, 27). Since resistant viral variants are likely to emerge in vivo, they should be monitored during the course of current clinical trials with A-77003 or related C_2 -symmetric inhibitors. Better understanding of mutations that confer increased drug resistance is important for the future development of other protease inhibitors.

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